Investigating Patterns of Mitochondrial DNA Inheritance Using New Zealand Chinook Salmon (*Oncorhynchus tshawytscha*) as a Model Organism

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ABSTRACT

The laws for the inheritance of animal mitochondrial DNA differ from those revealed for nuclear DNA. In contrast to nuclear genes, animal mitochondrial DNA (mtDNA) is predominantly inherited through the maternal line and is typically assumed to be non-recombining. The absence of both paternal transmission (hereafter: paternal leakage) and heterologous recombination of mtDNA are assumed to be key characteristics of mitochondrial DNA inheritance, which has enabled evolutionary models to be much simpler than those needed for the interpretation of nuclear DNA. However, recent revelations of paternal leakage in the animal kingdom challenge our current knowledge about mtDNA inheritance and the utility of mtDNA as a molecular marker. The occurrence of paternal leakage potentially introduces new haplotypes into populations and therefore impacts on the interpretation of mtDNA analysis. To date, it is unclear whether the documented cases of paternal leakage are exceptions to the general rule or if these events occur more frequently than so far believed. If this event occurred at a measurable frequency, it is vital to implement such data into models of mtDNA evolution to improve the accuracy at which evolutionary relationships and times of divergence are estimated.

In this thesis, I aimed to provide an insight into the broader patterns of mtDNA inheritance using chinook salmon as a model organism. I first sought to delimit the frequency of paternal leakage in chinook salmon and further investigated two major mechanisms which are believed to limit paternal leakage: The many-fold dilution of paternal mtDNA by maternal mtDNA upon fertilization and the genetic bottleneck mtDNA is believed to be exposed to during early developmental stages.

A screen of roughly 10.000 offspring did not reveal the presence of paternal mtDNA within these samples delimiting the maximum frequency of paternal leakage in

this system to 0.18% (power of 0.95) and 0.27% (power of 0.99), suggesting that the occurrence of paternal leakage is most likely an exception to the general rule.

To infer the dilution of paternal mtDNA upon fertilization, I employed real-time PCR and determined the mtDNA content of salmon spermatozoa and oocytes to be 5.73 ± 2.28 and $3.15x10^9 \pm 9.98x10^8$ molecules per gamete, respectively. Accordingly, the estimated ratio of paternal to maternal mtDNA in zygotes is $1:7.35x10^8 \pm 4.67x10^8$. This estimate is 3 to 5 orders of magnitude smaller than the ratio revealed for mammals.

Consequently, and if the dilution acts as an efficient barrier against the transmission of paternal mtDNA, paternal inheritance of mtDNA per offspring will be much less likely in this system than in mammals. To estimate at what probability the diminutive contribution of paternal mtDNA in zygotes is potentially inherited to offspring, I determined the size of the bottleneck acting on mtDNA during both embryogenesis and oogensis by examining the transmission of mtDNA variants to offspring and oocytes within a pedigree of heteroplasmic individuals. The number of segregating units (mtDNAs) between a mother's somatic tissue and oocytes was estimated to be 109.3 (median = 109.3; 62.4 < NeOog < 189.6; 95% confidence interval) and from a mother's soma to offspring's soma 105.4 (median = 105.4; 70.3 < NeEmb < 153.1; 95% confidence interval). Detected variances in allele frequency among oocytes were not significantly different from those in offspring, strongly suggesting that segregation of mtDNA occurs during oogenesis with its completion before oocyte maturation. However, considering a ratio of roughly $1:7.35x10^8$ for paternal to maternal mtDNA in zygotes and that approximately 109.3 (N_eOog) of the mitochondrial genomes present in zygotes are ultimately inherited to offspring, the probability for paternal mtDNA to be transmitted to offspring is in round terms

$1.0x10^{-11}$/paternal mtDNA molecule.

In summary, the results presented in this thesis document the presence of efficient barriers to prohibit the inheritance of minor allele contributions, such as paternal mtDNA, to offspring. These results strongly suggest that paternal leakage is an exception to the general rule. Furthermore, in comparison to studies undertaken in mammals, my results indicate that mechanisms in place to prevent paternal leakage may be unequally efficient among different animal taxa, reflecting differences in life traits, such as gamete morphology, gamete investment and reproductive strategies.

Nonetheless, by the means of the dilution effect in zygotes and the genetic bottleneck during oogenesis, the occurrence of paternal leakage might be simply a quantitative phenomenon and cannot be excluded *per se*. The increasing number of documented cases of paternal leakage clarifies that its occurrence must be considered when applying mtDNA as a genetic marker. Furthermore, for species in which mtDNA inheritance can be confirmed to be purely random, theoretical frequencies of paternal leakage can be inferred and potentially implemented into models of mtDNA evolution.

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Chapter 1

1 General Introduction

1.1 Mitochondria

Mitochondria are commonly known as the cellular power plants of eukaryotic cells. This terminology is due to their function as energy producing cell compartments. The process of energy synthesis in the form of adenosine-triphosphate (ATP), the main energy form in cells, is driven by an H^+-gradient generated between the matrix and the inner membrane of mitochondria and is catalyzed by oxidative phosphorylation (OXPHOS; Mitchell 1979). Mitochondria originate from a former free-living α-proteobacterium which was engulfed ~1.5 billion years ago by a heterotrophic unicellular organism (John et al. 1975; Margulis 1981; Olsen et al. 1994; Sicheritz-Ponten et al. 1998; Cavalier-Smith 2006). Capable of oxidative phosphorylation, a property useful to the host cell, the bacterium was stabilized inside the host and reduced to an organelle over time, a process described by the endosymbiont theory (Margulis 1981). The persistence of mitochondria over time as semi-autonomous cell compartments is believed to separate and protect the host DNA from DNA damage and cell damage in general, caused by the release of free radicals generated by the OXPHOS pathway during oxidative activity (Rand 1994; Lenaz 1998; Raha et al. 2000).

1.2 The mitochondrial genome, its inheritance and its utility as molecular marker

The majority of the protein-coding genes necessary to maintain mitochondrial functionality have been transferred to the host nucleus by horizontal gene transfer (Blanchard et al. 2000). Out of the estimated 3000-5000 genes originally present in the genome of the engulfed organism (Boussau et al. 2004), on average, 37 genes remain encoded in the

animal mitochondrial genome (Wolstenhome 1992). These genes typically encode for 22 transfer RNAs, two ribosomal RNAs and 13 subunits of the OXPHOS pathway (Wolstenholme 1992). The gene content of the mitochondrial genome is highly conserved within the animal kingdom (in regard to gene content) and generally arranged in a single but multi-copy circular double-stranded molecule, generally varying between 15 to 20 kilobases (kb) in length (Wolstenholme 1992).

Due to the endosymbiotic origin, the laws for the inheritance of mitochondrial genes differ from those revealed for nuclear DNA (Birky 2001). In contrast to nuclear genes, mtDNA is assumed to be inherited exclusively through the maternal line (Hutchison et al. 1974; Giles et al. 1980; Birky 1995). Why mtDNA follows this strict maternal mode of transmission is unknown and subject to speculation, with key points summarized in two major hypotheses.

The first hypothesis focuses on systems revealing oogamy, in which non-motile eggs are fertilized by motile sperm. As cell movement is typically dependant on mitochondrial activity and mitochondrial activity is associated with mtDNA damage, sperm mtDNA may accumulate potentially lethal mutations during the time of oxidative activity to reach the egg (Rand 1994; Lenaz 1998). In such cases, the inheritance of maternal mtDNA might be favored as eggs are assumed to maintain a quiescent metabolism and maternal mtDNA should be still in a relatively pristine state (Allen 1996; Bromham et al. 2003). An alternative hypothesis is based on the idea of selfish mitochondria (Hastings 1992). If mitochondria were inherited from both parents, fertilized zygotes would contain two genetically distinct populations of mitochondria. The presence of two genetically distinct mtDNA haplotypes allows for natural selection and might occur at the expense of single individuals and subsequently of whole populations. For example, if one haplotype would increase its own rate of replication (e.g. due to a major deletion), and is associated with

reduced metabolic activity, its frequency might nonetheless increase and potentially spread within a population, consequently reducing individual and population fitness (Hastings 1992; Hurst 1996).

The mechanisms which either prevent paternal inheritance or promote maternal inheritance of mtDNA are not fully understood but it appears that there is some variation between species (Birky 1995). In tunicates, for example, it has been shown that the mitochondria-containing mid-piece of sperm does not enter the egg upon fertilization, prohibiting the inheritance of paternal mtDNA *per se* (Ursprung und Schabtach 1965). In mammals instead, sperm mitochondria enter the egg (Ankel-Simons and Cummins 1996) but are ubiquitinated upon entry and subsequently degraded (Sutovsky et al. 1999). In addition to such species-specific mechanisms, it is believed that the inheritance of paternal mtfDNA is further inhibited by two major mechanisms: Firstly, it is assumed that if paternal mtDNA enters the egg, it is vastly outnumbered by maternal mtDNA as oocytes contain hundreds of thousands mtDNA molecules whereas sperm generally contain just a few copies of the mitochondrial genome (Birky 1995; Ankel-Simons and Cummins 1996, Bromham et al. 2003). Secondly, the presence of a genetic bottleneck during embryogenesis or oogenesis is believed to exclude rare mtDNA haplotypes, such as paternally derived mtDNA, from transmission to offspring (Birky 1995; Ankel-Simons and Cummins 1996; Marchington et al. 1998). Both mechanisms are believed to be corner stones of mitochondrial inheritance which prevent paternal mtDNA from transmission.

However, as a consequence of strict maternal inheritance, the absence of both paternal transmission (hereafter: paternal leakage) and heterologous recombination are believed to be key characteristics of mtDNA inheritance (Brown et al. 1979; Birky 1995; Ballard and Dean 2001). These characteristics combined with the small size of the mitochondrial genome, its high copy number (Wiesner et al. 1992) and higher mutation

rate (compared to nuclear genes; Brown et al. 1979) has led mtDNA based analysis to become a powerful tool in population genetics, phylogenetics and phylogeography (Birky 1983; Coyer et al. 2005; Avise 2000; Slate and Gemmell 2004; Lockridge Mueller 2006). Considering mtDNA as non-recombining and maternally inherited, it is possible to construct unambiguous genealogies from a sample of mtDNA sequences. Furthermore, assuming that present mutations arose independently, it is possible to date single events in such genealogies using a molecular clock (assuming a constant rate of molecular evolution; Bromham and Penny 2003). Such analyses have been widely used to elucidate the evolutionary history of numerous species and have been employed to determine, for example, the origins and the global expansion of modern humans (Pakendorf and Stoneking 2005; Cann et al. 1987; Ingmann et al. 2000, Maca-Meyer et al. 2001; Vigilant et al. 1991). Employing mtDNA analyses and applying a molecular clock, the age of our most recent common ancestor (MRCA) has been estimated to be ~171,000 years (Ingmann et al. 2000). Further analyses of human mtDNA haplotypes from individuals representing major linguistic phyla further support the idea that modern humans are likely to have originated from Africa and subsequently colonized the world (Pakendorf and Stoneking 2005; Cann et al. 1987; Maca-Meyer et al. 2001; Vigilant et al. 1991).

1.3 Paternal transmission of mtDNA (paternal leakage)

Paternal leakage and recombination of mtDNA have been recognized to occur in plant, fungal and protist species, leaving the animal kingdom as the last stronghold of strict maternal inheritance (Gillham 1994). In recent years however, there has been accumulating evidence for both paternal leakage and recombination in a wide range of animal species (Table 1.1). Paternal leakage of mtDNA has been documented in at least 15 species and

mtDNA recombination in at least 11 species, covering highly divergent taxa such as mammals, arthropods, molluscs, nematodes, birds and fish (Table 1.1). Furthermore, in several bivalves (*Mytilis spec.* and *Tapes Philippinarum*), it is now known that biparental inheritance is actually the rule (Passamonti and Scali 2001; Breton et al. 2007). Although apparently gender-limited (leaked paternal mtDNA contributes exclusively to the male germ line in offspring; Skibinski et al. 1994; Passamonti and Scali 2001), these findings clearly challenge the paradigm of strict maternal inheritance and indicate that our current knowledge of mtDNA inheritance might be incomplete.

Whereas first discoveries of either paternal leakage or recombinantion of mtDNA were regarded as extraordinary exceptions and were largely ignored, the increasing number of revealed cases in the animal kingdom in recent years questions our current understanding of mitochondrial inheritance and to some extent the applicability of mtDNA as a molecular marker.

If paternal leakage occurrs and paternal mtDNA is transmitted to offspring, this process would introduce new mtDNA haplotypes into the overall mtDNA pool of single populations (given maternal and paternal mtDNA haplotypes are genetically distinct). Furthermore, once inherited, it is possible that paternal and maternal mtDNA recombine during early embryogenesis after fusion of paternal and maternal mitochondria, creating even greater haplotype diversity in descending offspring (Eyre-Walker 2000; Rokas et al. 2003). Interestingly, it has been shown that fusion and fission of mitochondria are common during different developmental stages, including embryogenesis (Smith et al. 1993; Chan 2006), and it has been further shown that the molecular tool kit to catalyze both heterologous and homologous recombination is widespread in the animal kingdom (Thyagarajan et al. 1996; Santel and Fuller 2001; Yaffe 1999; Lakshmipathy and Campbell 1999).

The introduction of additional haplotypes and informational sites (through paternal leakage), and the formation of new haplotypes (through heterologous recombination), may have significant impact on the accuracy of sequence analysis and interpretation (Schierup and Hein 2000, Eyre-Walker 2000; Bromham et al. 2003; Slate and Gemmell 2004). Schierup and Hein (2000) concluded after simulating the influence of varying degrees of recombination on tree-based phylogentic analyses, that the presence of recombination may lead to (i) underestimation of the time of our MRCA, (ii) underestimation of the amount of recent divergence, (iii) overestimation of number of mutations, (iv) apparent signs of exponential growth, (v) loss of molecular clock, and (vi) apparent substitution rate heterogeneity among sites (see also: Eyre-Walker 2000, Slate and Gemmell 2004). In other words, the presence of paternal leakage and subsequent recombination could potentially average the evolutionary distance between mtDNA sequences by 'blending' or combining informational sites from originally distinct haplotypes. Consequently, if a molecular clock is applied, dates of divergence are likely to be underestimated and individuals or species appear more closely related as all sequences reveal a higher degree of similarity (Eyre-Walker 2003). For example, it has been shown that mtDNA sequences affected by recombination would still support the idea of an African origin of modern humans but the age of our MRCA would be significantly underestimated (Schierup and Hein 2000). More precisely, the age of our MRCA is estimated to be ~171,000 years if recombination is ruled out and ~340,000 years if the applied model accounted for the potential presence of recombinant sequences, changing our view of human prehistory (Ingmann et al. 2000; Eyre-Walker 2003; Schierup and Hein 2000; Slate and Gemmell 2004).

Considering the increasing number of cases revealing either paternal leakage or recombination of mtDNA and their potential impact on the utility of mtDNA as a molecular marker, it is necessary to further investigate the mechanisms of mitochondrial

inheritance to allow for accurate sequence analyses and interpretation. More precisely, it needs to be clarified if the detected cases of paternal leakage and recombination are exceptions to the general rule or if these events occur more frequently than so far believed.

To date, the frequency of paternal leakage remains ambiguous as most evidence for this phenomenon is derived from anecdotal observation, or interspecific crossing experiments, which by definition are uncommon in nature and even more importantly, are assumed to promote paternal transmission of mtDNA (Sutovsky et al. 2000; Sherengul et al. 2006). Moreover, failure to detect paternal leakage in many cases might be simply due to small sample sizes investigated in those studies. It is therefore desirable to conduct such studies with larger sample sizes and under more natural conditions, i.e. with exclusion of potentially misleading factors such as inbreeding, back-crossing and hybridization. Also, as the mechanisms to prohibit paternal leakage are not fully understood, it is desirable to conduct further research towards the characterization of such mechanisms (e.g. dilution of paternal mtDNA in zygotes, genetic bottleneck during embryogenesis/oogenesis), to estimate with what probability, if measurable, paternal mtDNA is likely to be inherited to offspring. Such studies would not only contribute to the better understanding of mtDNA inheritance and potentially allow for implementation of such factors in evolutionary models but would also help to evaluate the significance of recent revelations of paternal leakage and recombination in the animal kingdom.

Table 1.1: Documented cases of paternal leakage and recombination of mtDNA in the animal kingdom.

Species	Paternal leakage	Recombination	Researcher
Human (*Homo sapiens*)	yes	yes	Schwartz and Vissing 2002; Kraytsberg et al. 2004; Zsurka et al. 2004
Primate (*Macaca nemestrina*)	no	yes	Piganeau 2004
Mouse (*Mus spretus*)	yes	no	Gyllensten et al. 1991; Shitara et al. 1998
Vole (*Microtus longicaudus*)	no	yes	Piganeau 2004
Sheep (*Ovis aries*)	yes	no	Zhao et al. 2004
Great tit (*Parus major*)	yes	no	Kvist et al. 2003
Anchovy (*Engraulis encrasicolus*)	yes	no	Magoulas and Zouros 1993
Perch (*Micropterus salmoides*)	no	yes	Piganeau 2004
Crucian Carp (*Carassius aurathus* x *Cyprinus carpio*)	yes	yes	Guo et al. 2006
Flatfish (*Platichthys flesus*)	no	yes	Hoarau et al. 2002
Salmon (*Salmo salar x S. trutta*)	no	yes	Ciborowski et al.
Mussel (*Mytilis edulis*, *M. galloprovincialis*)	yes	yes	Zouros et al. 1992; Breton et al. 2007; Theologidis et al. 2007
Scorpion (*Buthus spp., Mesobuthus spp*.)	yes	yes	Gantenbein et al. 2005
Lizard (*Chlamydosaurus kingii*)	yes	yes	Ujvari et al. 2007
Honeybee (*Apis mellifera L*.)	yes	no	Meusel and Moritz 1993
Silkmoth (*Antheraea proylei*)	yes	no	Arunkumar et al. 2006
Fruitfly (*D. simulans x D. mauritiana*)	yes	no	Kondo et al. 1990; Sherengul et al. 2006
Cicada (*Magicicada septendecim x M. cassini x M. septendecula*)	yes	no	Fontaine et al. 2007
Nematode (*Meloidogyne javanic, Globodera pallida, Bursaphelenchus conicaudatus*)	no	yes	Lunt and Hyman 1997; Armstrong et al. 2007; Piganeau 2004

1.4 New Zealand chinook salmon as a model organism

New Zealand Chinook salmon are an ideal experimental model system in which to investigate the patterns of mtDNA inheritance as their biology, husbandry and genetics are well documented (Pennell and Barton 1996; Quinn 1996; Wilhelm et al. 2003). More importantly, single females produce between 3000 and 5000 eggs (Pennell and Barton 1996; Kinnison 1998), providing the sample sizes necessary to reveal potentially rare events, such as paternal leakage. Furthermore, male and female gametes can be harvested in uncomplicated fashion and fertilization experiments can be conducted externally using the dry method, allowing a high degree of manipulation (Huet 1970; Pennell and Barton 1996). Individuals used in this study were part of a hatchery population at the NIWA Silverstream hatchery (www.niwascience.co.nz). All individuals of this population are semi-wild returns, i.e. fish were reared at the hatchery and then released into the wild. Once matured, these individuals return to the hatchery for spawning. Using this system to investigate patterns of mitochondrial inheritance, such examinations can be undertaken intraspecifically and without potentially misleading factors, such as high degrees of back-crossing, inbreeding or hybridization.

Previous work on this hatchery population revealed the presence 36 single nucleotide polymorphisms (SNPs) within the mitochondrial genes *mt-nd1* to *mt-nd5* (Table 1.2; Metcalf et al., unpublished data) and three main mtDNA lineages (X, Y, Z, Figure 1.1) Each of the three main mtDNA lineages harbors at least one SNP which is unique for the corresponding haplogroup (diagnostic SNP). The presence of diagnostics SNPs allows for unambiguous determination of mtDNA origin among offspring (i.e. paternally or maternally derived) between these lineages and is therefore suitable to observe and investigate patterns of mitochondrial inheritance in this system. In addition, two SNPs have

been identified to be heteroplasmic sites (see Table 1.2). These heteroplasmic sites are synonomous and have been identified by standard Sanger sequencing and have been confirmed by cloning and subsequent Sanger sequencing. These heteroplasmic sites can be used to monitor the inheritance of heteroplasmy and to determine the genetic bottleneck acting during oogenesis and/or embryogenesis.

Table1.2: Summary of mitochondrial SNPs (nucleotide position) and main mtDNA lineages with associated haplotypes detected within the Silverstream hatchery. Main haplotypes are shown with subhaplotypes indicated underneath. The most common SNP pattern for each mtDNA lineage is depicted first and deviations from this common pattern constitute the subhaplotypes underneath.

		mt-nd1							***mt-nd2***							***mt-nd3***			***mt-nd4***														***mt-nd5***					
mtDNA lineages and haplotypes		3957	4113	4149*	4152	4316*	4698	4747	5285	5393	5425	5539	5662	5842	5897	10650	10725	10949	11134	11209	11227	11612	11649	11756	12033	12381	12522	12537	12567	12642	12942	12963	12990	13092	14272	14526	14687
	X	a	a	g	a	t	t	c	a	a	a	a	t	g	c	g	g	g	g	a	t	g	c	a	c	a	t	g	c	g	t	g	g	g	g	t	g
			g		g		c															a			t	g		a	t							c	
																						a						a	t								
	Y	a	g	g	g	t	t	a	a	a	a	a	t	g	c	a	a	a	g	a	t	a	c	a	c	g	t	a	t	g	t	a	a	a	a	c	g
							c	c														g																
				a/g			c	c						a			g									t										g			
			a				t																									g		g				
			a		a		t																															
																											c										
	Z	a	a	g	g	t	t	c	g	a	a	a	t	g	t	a	g	a	a	a	t	a	a	g	c	a	t	a	t	a	t	g	a	g	g	t	a
									a			g												a													g
			g				c		a			g												a													g
									a						c								c	a													g
						c/t																																
						c/t									t																							

Diagnostic SNPs are highlighted in orange; * heteroplasmic sites; nucleotide positions according to NCBI: NC_002980)

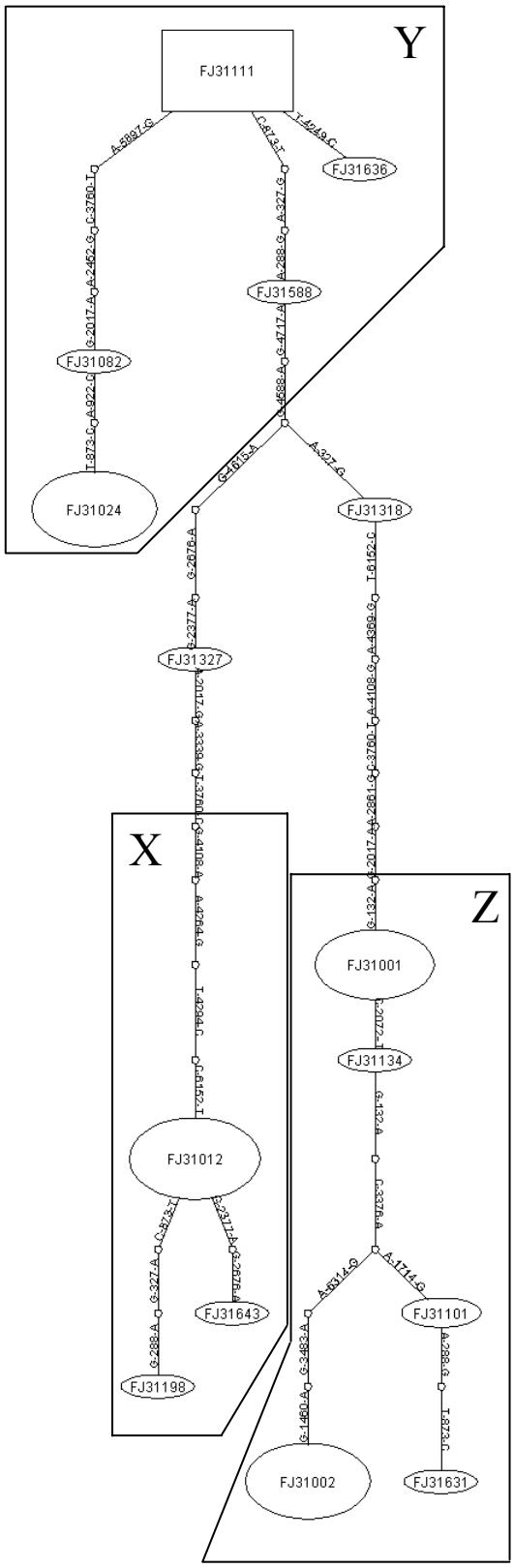

Figure 1.1: TCS haplotype network connecting 15 haplotypes detected in a sample of 62 individuals. Haplotypes are based on 6397bp of mtDNA sequence information within the genes *mt-nd1* to *mt-nd5* (connecting limit of the network: 35 steps). Distinct mtDNA lineages (W, X, Y, Z) are based on nested clade analysis and were identified from three-step clades.

1.5 Aim of thesis

Recent revelations of paternal leakage and recombination in the animal kingdom challenge our current knowledge about mtDNA inheritance and therefore the utility of mtDNA as a molecular marker. To elucidate whether the documented cases of paternal leakage are exceptions to the general rule or if this event occurs at low frequencies which remain to be determined, I first delimit the frequency of paternal leakage in New Zealand chinook salmon in this thesis. To further understand better mtDNA inheritance, I investigate two major mechanisms believed to prohibit paternal leakage and subsequently mtDNA recombination. I determine the mtDNA content of salmon gametes to describe the dilution effect of paternal mtDNA upon fertilization and examine the inheritance of mtDNA heteroplasmy to describe the genetic bottleneck acting during embryogenesis. The results gained in these objectives enable me to evaluate the significance of recent revelations of paternal leakage and recombination and their significance for mtDNA analyses.

The main chapters of this thesis (Two, Three, Four, Five and Six) have been written as self-contained manuscripts for submission to international scientific journals, which has resulted in a certain degree of repetition.

In Chapter 2, I describe the development of a novel experimental approach designed for the detection of small allele contributions in heteroplasmic DNA samples. The development of this methodology was crucial for my thesis as this approach combines an increased sensitivity for paternal mtDNA compared to conventional methods and the capability to process large sample sizes necessary to detect potentially rare events, such as paternal leakage. This chapter has been published in BioTechniques (Wolff and Gemmell 2008).

In Chapter 3, I describe how I applied this novel experimental approach to screen for the potential presence of paternal leakage within the samples investigated and present the results, enabling me to delimit the frequency of paternal leakage in New Zealand chinook salmon. This chapter has been accepted for publication in Genetics (pending upon revision, requested changes will affect the manuscripts length but not its content).

In Chapter 4 and Chapter 5, I present the work I have undertaken to estimate the mtDNA content in chinook salmon gametes to describe one of the major mechanisms preventing paternal leakage: The dilution of paternal mtDNA by maternal mtDNA upon fertilization. In Chapter 4, I present the work undertaken to estimate the number of mtDNA molecules per sperm in New Zealand chinook salmon. This manuscript has been accepted for publication in Biology of Reproduction (pending upon revision; manuscript has been revised and resubmitted). In Chapter 5, I present the work conducted to estimate the mtDNA content of chinook salmon oocytes and the description of the dilution effect of paternal mtDNA upon fertilization. This manuscript has been accepted for publication in Heredity (pending upon revision, requested changes will affect the manuscripts length but not its content).

In Chapter 6, I extend the work from Chapter 4 and Chapter 5, and determine the genetic bottleneck acting during embryogenesis to estimate how many mtDNA molecules present in fertilized zygotes are likely to be inherited to progeny. This work is still in progress and the chapter is in preparation for submission.

In Chapter 7, I combine the results from Chapter 4, 5 and 6, allowing me to estimate the probability at which paternally derived mtDNA is inherited to offspring and I will further discuss and conclude all other work undertaken in this thesis.

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Chapter 2

2 Combining Allele-Specific Fluorescent Probes and Restriction Assay in Real-Time PCR to Achieve SNP Scoring Beyond Allele Ratios of 1:1000*

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2.1 Abstract

TaqMan®-nuclease assays are widely employed for the qualitative detection of single nucleotide polymorphism (SNPs) and the determination of biallelic states in pooled or heterozygous DNA samples. These assays are highly specific, reproducible and suitable for high-throughput approaches. A crucial limitation of this method, and others, is the detection of minor allele frequencies with detection limits of generally 3% to 9% for minor allele contributions. Here, we describe the combination of customized TaqMan®-nuclease assay and allele-specific restriction to increase the sensitivity of this method, allowing the qualitative detection of allele contributions as low as 0.05%.

2.2 Introduction

Single nucleotide polymorphisms (SNPs) are estimated to occur at a frequency of 1 SNP per 1-2 kb (Sachidanandam et al. 2001). Due to this high abundance throughout the genome, SNPs find a wide range of applications as molecular markers in, for example, forensics (Brandstätter et al. 2003), medical diagnostics (Wei et al. 2002) and molecular epidemiology (Martin et al. 2000). A wide range of techniques have emerged to score SNPs qualitatively or quantitatively (Kwok 2003) including: TaqMan® -nuclease assay (Breen et al. 2000), molecular beacon assay (Mhlanga and Malmberg 2001), fluorescent labeled locked nucleic acids (LNA) (Johnson et al. 2004), pyrosequencing (White et al. 2005), multiplex quencher extension (Rudi et al. 2006) and polymorphism ratio sequencing (Blazej et al. 2003). Although these methods allow the detection of alleles in pooled or heterozygous samples if present at frequencies of 3-9%, the sensitivity of these methods is

limited and often insufficient for many applications. In disease-association studies where allele contributions in whole-tissues of less than 2% can be crucial (Chinnery et al. 2001; Liu et al. 2004) or in biological studies where the contribution of minor alleles in heteroplasmic mitochondrial DNA samples can be as low as 0.01% (Gyllensten et al. 1991), such allele frequencies cannot be detected without a tradeoff in both experimental design and sample throughput (Blazej et al. 2003; White et al. 2005; Rudi et al. 2006).

TaqMan®-nuclease assays are well-established and commonly used for SNP detection and genotyping studies (De la Vega et al. 2005; Fornage and Doris 2005). Using this methodology, the detection of SNPs goes hand in hand with the amplification of a single short DNA sequence containing the SNP site. Fluorescent probes differentiating between the two alleles bind at the SNP site located between the two primer sites and are degraded during amplification by the exonuclease activity of *Taq* polymerase, releasing a fluorescent reporter dye. The amplification of a single DNA sequence to subsequently differentiate among allelic states is convenient and allows high-throughput, but the downside of this design is the use of the same primer set for the amplification of both alleles. The use of just one primer pair leads automatically to a competing amplification between these alleles. If one allele is present at a lower frequency than the other, the amplification is likely to be dominated by the allele at higher frequency. Thus, the amplification of the second allele with lower copy number is suppressed and often not detectable.

2.3 Material, Methods and Results

To minimize this effect, we combined allele-specific restriction with TaqMan® -nuclease assay, in order to suppress the amplification of the dominant allele and to allow the qualitative detection of small allele contributions. To evaluate the power of this combined approach we compared the detection limit for alleles occurring at low frequencies of the conventional TaqMan®-nuclease assay to the detection limit of the combined TaqMan/restriction approach.

The investigated SNP was a G/A polymorphism (with A = wildtype and G = mutant) in the salmon *mt-nd1* gene at nucleotide position 3957 of the mitochondrial genome (NCBI: NC_002980). This SNP has been detected in previous work within individuals of a hatchery population of chinook salmon (data not published).

To evaluate the sensitivity of both approaches, we extracted DNA from fin clips of 20 salmon using the DNeasy Blood & Tissue kit (Qiagen GmbH, Hilden, Germany). We amplified and sequenced a 314 bp fragment of the mitochondrial genome of these samples using the primers ND1aF and ND1R1 to identify individuals harboring either the mutant (G) or the wildtype version (A) of the SNP (for primer sequences see Table 2.1). DNA purity was checked using a ND-1000 NanoDrop (NanoDrop Tech., Wilmington, USA) and two-fold serial dilutions were prepared for mutant DNA samples (starting concentration: 4.5ng/µl, 13 dilution steps), aliquoted and stored at -20ºC. As a measure of accuracy, we derived a standard curve from real-time PCR amplifications applying the TaqMan®-nuclease assay on this dilution series. The amplification was performed with an efficiency of 98.5% (data not shown), confirming the accurate dilution of template DNA, which was then used for DNA pool construction. Mutant/wildtype DNA pools were prepared by

adding the two-fold dilution series of mutant DNA to a constant remaining DNA solution of wildtype DNA (9ng), resulting in ratios of 1:2 to 1:8192.

The enzyme restricting the SNP allele-specific (*Ava*II, motif: GGWCC, with W = A or T), was identified using NEBcutter V2.0 (New England Biolabs Inc., Ipswich, USA). Digests were performed for two hours at 37ºC using 1 unit of enzyme per 1µg DNA, followed by 20 minutes heat inactivation at 65ºC. Restrictions were applied on both wildtype and mutant DNA samples and were performed prior to the preparation of dilution series and pool construction.

Genotyping was performed using TaqMan®-nuclease assays (Applied Biosystems, Foster City, USA). Experiments were carried out using the Mx3000P Q-PCR system (Stratagene, Garden Grove, USA) and consisted of: initial denaturation for 3 min at 95ºC, followed by 55 cycles of denaturation at 95ºC for 30 sec and combined annealing and elongation at 63ºC for 1 min (for sequence information of primers and probes see Table 2.1). The change in fluorescence was measured at the end of each extension step. All samples were tested in triplicate and results were analyzed using the software MxPro, supplied with the instrument.

To evaluate the level of background fluorescence due to misannealing of the TaqMan probe, positive controls homoplasmic for wildtype DNA were included in each experiment. Endpoint measurements of fluorescent signal for the mutant probe on wildtype DNA were set as default background fluorescence. All samples were background-corrected and considered positive for mutant DNA detection if sample fluorescence was two standard deviations above background fluorescence. Automatic genotyping followed after background-correction using a dual scatter plot.

Once DNA samples with known ratios of mutant to wildtype DNA were prepared, we applied a customized TaqMan®-nuclease assay to DNA samples that varied from 1:2 to

1:512 mutant to wildtype DNA to evaluate the detection limit of the conventional method. With this assay we detected the mutant allele in ratios as low as 1:64, corresponding to ~1.6% of the minor allele contribution (Figure 2.1). Fluorescence signals for ratios ≤1:128 were below background. After background-correction, all samples were automatically genotyped using a dual scatter plot (Figure 2.2). Samples with a ratio of 1:2 to 1:64 were genotyped as heteroplasmic, whereas samples ≤1:128 and the wildtype control were genotyped as homoplasmic for the wildtype allele (Figure 2.2). To increase the sensitivity of this method we then combined the TaqMan®-nuclease assay with allele-specific restriction. With this approach the amplification of the dominant wildtype allele was repressed, enabling the qualitative detection of the mutant allele in ratios as low as 1:2048 (Figure 2.3). Compared to the conventional approach, fluorescence signals were approximately 50% higher in total fluorescence, indicating a higher efficiency (i.e. higher amounts of final PCR product) for the amplification of the mutant allele. Ratios ≤1:4096 were below background. Samples with ratios of 1:2 to 1:2048 were genotyped as heteroplasmic and samples 1:4096, 1:8192 and wildtype control were genotyped as homoplasmic for wildtype DNA (Figure 2.2).

Table 2.1: Sequences of primers and probes.

Primer/probe	Sequence 5` to 3`	Basepairs
Forward primer ND1aF	GGTAATTGCGAGAGGCCTAA	20
Reverse primer ND1R1	GTAAGGGCAAGTATGGGTGT	20
Forward primer (TaqMan)	AACGAAAAGTCCTTGGGTATATGCA	25
Reverse primer (TaqMan)	CCGTCTGCGATAGGTTGCA	19
Wildtype probe	TCGAAAAGGACCAAACA	17
Mutant probe	TCGAAAAGGGCCAAACA	17

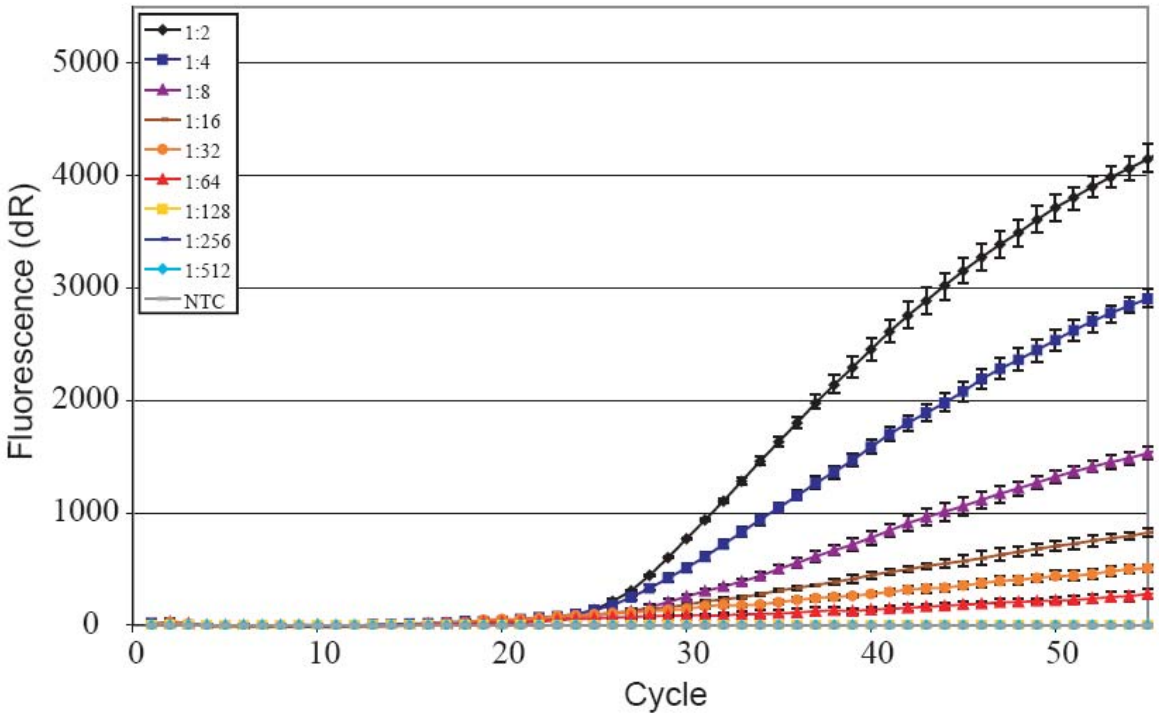

Figure 2.1: Conventional TaqMan assay. Results for the detection of the mutant allele of the conventional TaqMan® nuclease assay applied on DNA ratios ranging from 1:2 to 1:512 mutant to wildtype DNA. Background-corrected relative fluorescence (dR) plotted against cycle number. Signal of ratios of 1:128 to 1:512 are not visible as plots are congruent with plots of NTC. NTC = no template control.

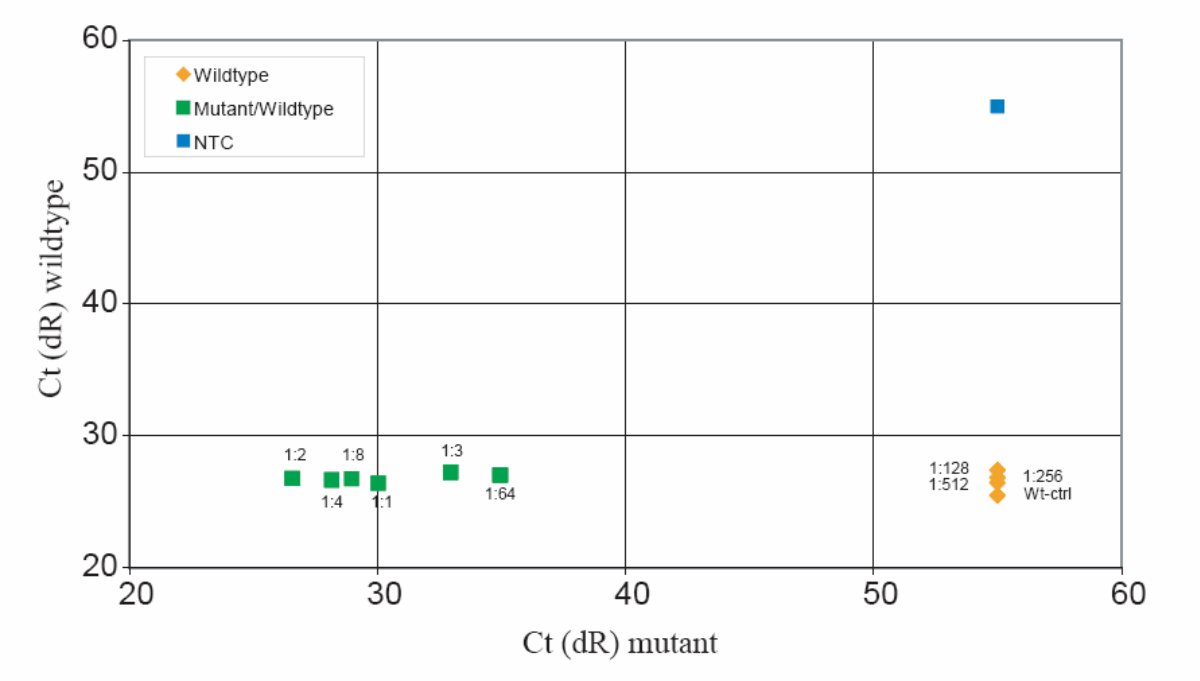

Figure 2.2: Dual scatter plot of conventional TaqMan assay. Results of the conventional TaqMan® nuclease assay applied to DNA ratios ranging from 1:2 to 1:512 mutant to wildtype DNA. Threshold cycle (Ct) of the mutant allele amplification plotted against the threshold cycle (Ct) of the wildtype allele amplification. NTC = no template control; Wt-ctrl = wildtype control.

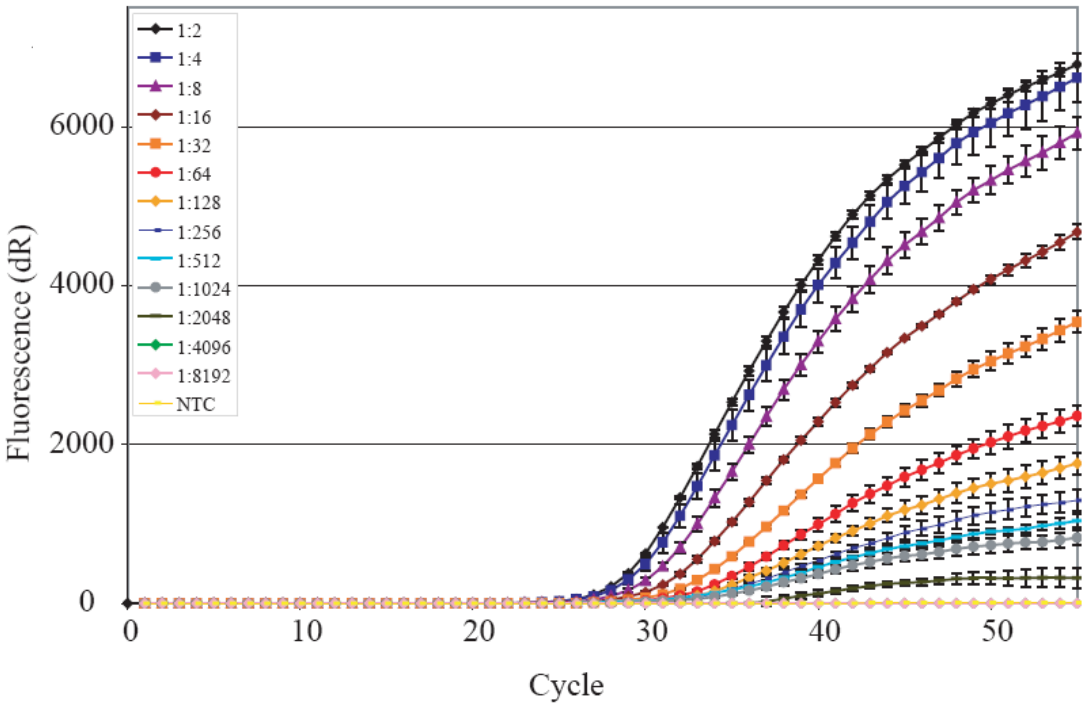

Figure 2.3: TaqMan assay combined with allele-specific restriction. Results of the modified TaqMan/restriction assay applied to DNA ratios ranging from 1:2 to 1:8192 mutant to wildtype DNA. Amplification plots for the mutant allele. Background-corrected relative fluorescence (dR) plotted against cycle number. Signal of ratios 1:4096 to 1:8192 are not visible as plots are congruent with plots of NTC. NTC = no template control.

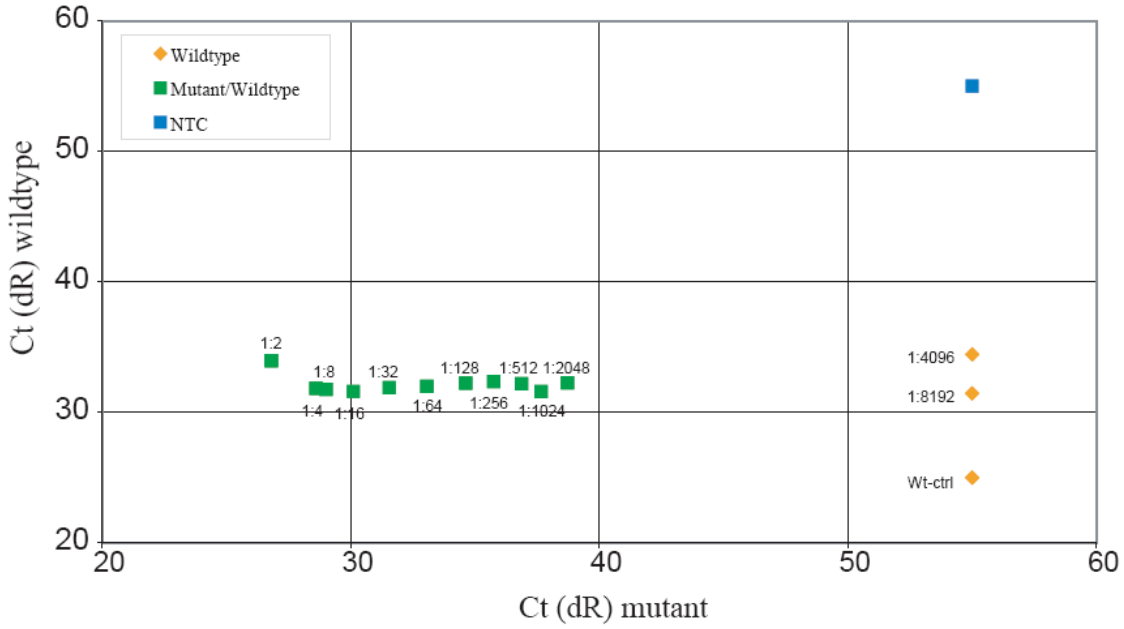

Figure 2.4: Dual scatter plot of TaqMan assay combined with allele-specific restriction. Results of the modified TaqMan/restriction assay applied to DNA ratios ranging from 1:2 to 1:8192 mutant to wildtype DNA. Threshold cycle (Ct) of the mutant allele amplification plotted against the threshold cycle (Ct) of the wildtype allele amplification. NTC = no template control; Wt-ctrl = wildtype control.

2.4 Discussion

With the incorporation of a restriction step prior to the conventional TaqMan®-nuclease assay, the sensitivity of this particular assay was increased 32 fold, allowing the qualitative detection of minor allele contributions as low as 0.05%. However, restricting the wildtype allele did not lead to a complete inhibition of wild type amplification and therefore an absence of increasing endpoint fluorescence for the wildtype probe. It is likely that the residual relative fluorescence for the wildtype allele after restriction was caused by incomplete digests of template DNA. Furthermore, it is also conceivable that restricted wildtype DNA might still serve as a template for the wildtype TaqMan probe to anneal. The restriction site of the template DNA corresponds to the central base of the TaqMan probe, so it is possible that the probe anneals over half of its length to the template DNA. Once annealed the probe can still be degraded through the exonuclease activity of *Taq* polymerase separating quencher and reporter which are located at 5` and 3` end without degrading the probe over the whole length. This process is not likely to occur in an efficient manner but might contribute to the residual fluorescence of the wildtype probe.

Nevertheless, we have demonstrated that the amplification of a dominant allele can be repressed through restriction, increasing the sensitivity of the TaqMan®-nuclease assay by at least 32 times. This increase could potentially prove valuable in, for example, cancer research where the detection of low level point mutations can serve as indicator for the developing disease (Jackson and Loeb 1998). The malignant transformation of single cells over time caused by the accumulation of mutations often remains undetected using standard genotyping methods until proliferation of the affected cells increases the frequency of mutant alleles (Loeb 2001). In such cases, detecting the presence of low level

mutation can be important in regard to potential prognostic value and treatment (Jackson and Loeb 1998; Loeb 2001).

However, weaknesses of this approach are the loss of reliable quantification capability and the necessity to find a restriction enzyme that cuts the SNP site in an allele-specific manner. Generally, restriction enzymes are easily accessible and rich in the variety of motifs they recognize. An initial screen of 7 SNPs detected in chinook salmon mitochondrial DNA prior to this study revealed the availability of allele-specific restriction enzymes in 6 cases (see supplemental Table 2.2). Even though restriction enzymes might not be obtainable for all SNPs, we are convinced that this application will prove valuable in those cases where an allele-specific restriction enzyme can be found.

If reactions are carried out in multi-well PCR plates the combination of restriction assay and TaqMan assay can be readily applied with no significant tradeoff in throughput. Thus, this combined approach represents a valuable improvement for studies where the primary goal is the qualitative detection of alleles occurring at low frequencies and high sample-throughput are essential.

2.5 Acknowledgements

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2.6 Competing Interests Statement

The authors declare no competing interests.

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2.8 Supplemental Material

Table 2.2: Overview of SNPs and coresponding allele-specific restriction enzymes. Seven SNPs of chinook salmon mitochondrial DNA and corresponding restriction enzymes that cut single SNPs allele-specific. A screen for allele-specific restriction enzymes for the wildtype allele of each SNP revealed the availability of wildtype-specific restriction enzymes in six cases. Enzyme-specific recognition motifs are listed and cut sites indicated within the wildtype sequences

Nucleotide position	SNP	Allele	Sequence 5` to 3`	Restr. enzyme	Motif	Cuts
3957	G/A	wt	CTTCGAAAAGG[A]CCAAACATCGT	*Ava*II	GGWCC	wt
		mt	CTTCGAAAAGG[G]CCAAACATCGT			
4149	G/A	wt	GATCTTAACCT[A]GGGGTACTATTT	*Avr*II	CCTAGG	wt
		mt	GATCTTAACCT[G]GGGGTACTATTT			
4316	T/C	wt	GCGTGATTATCA[T]CACGGGGGGG	*BsaB*I	GAT(N)$_4$ATC	wt
		mt	GCGTGATTATCA[C]CACGGGGGGG			
5842	G/A	wt	AAACAAGAACT[G]CCACTACCTGC	-	-	-
		mt	AAACAAGAACT[A]CCACTACCTGC			
10650	G/A	wt	CTTAATTACAACA[G]TCATCACTAT	*HpyCH4*III	ACNGT	wt
		mt	CTTAATTACAACA[A]TCATCACTAT			
10725	G/A	wt	ATCTCCCCAGAC[G]CAGAGAAGTT	*Hga*I	GACGC	wt
		mt	ATCTCCCCAGAC[A]CAGAGAAGTT			
11649	C/A	wt	CTGGTCTCCCT[C]CAAATATTTCTA	*Mnl*I	CCTC	wt
		mt	CTGGTCTCCCT[A]CAAATATTTCTA			

Nucleotide positions according to NCBI: NC_002980; SNP in brackets; recognition site of enzymes underlined; motifs listed with W = A or T and N = A, C, G or T; wt = wildtype, mt = mutant

Chapter 3

3 Delimiting the Frequency of Paternal Leakage of Mitochondrial DNA in Chinook Salmon*

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3.1 Abstract

Animal mitochondrial DNA (mtDNA) is widely employed as a molecular marker due to its apparent clonal inheritance through the maternal line. However, accumulating evidence for paternal transmission (paternal leakage) across a wide range of taxa is challenging our understanding of mtDNA inheritance and its reliability as a molecular marker. Prior studies aiming to detect paternal leakage estimated the contribution of paternal mtDNA within single samples but failed to estimate how frequently paternal leakage might occur. Furthermore, samples investigated in these studies were derived from crossing regimes employing varying degrees of inbreeding and back-crossing, which are believed to promote the transmission of paternal mtDNA. Here, we undertook the first study to systematically estimate the frequency of paternal leakage within a species, under semi-wild conditions. We utilized crosses among divergent mtDNA types found within a wild return hatchery population of chinook salmon. We analyzed 10,082 offspring and assigned the mtDNA to either parent using quantitative TaqMan® assays. None of these samples revealed paternally transmitted DNA, limiting the maximum frequency of paternal leakage to 0.18% (power of 0.95) and 0.27% (power of 0.99).

3.2 Introduction

Animal mitochondrial DNA (mtDNA) is widely employed as a molecular marker in various disciplines of biology, such as population genetics, phylogeography and phylogenetics (Brown et al. 1979; Avise et al. 1987; Moritz et al. 1987). The utility of mtDNA as a genetic marker derives from its unique patterns of inheritance. In contrast to nuclear genes, mtDNA is predominantly inherited through the maternal line. Consequently, the absence of both paternal transmission (paternal leakage) and heterologous recombination of mtDNA are believed to be key characteristics of mtDNA inheritance (Birky 1995; Barr et al. 2005). These features combined with its small size (generally 15 to 20kb), high copy number and higher mutation rate (compared to nuclear genes) have greatly facilitated the investigation of complex genetic ancestries and phylogeographic or phylogenetic patterns (Birky et al. 1983; Birky 2001; Slate and Gemmell 2004).

In recent years however, there has been accumulating evidence for both paternal leakage and recombination to occur in a wide range of taxa within the animal kingdom. Both paternal leakage and recombination have been documented in at least 15 species. These species include mammals (Gyllensten et al. 1991; Shitara et al. 1998; Kajander et al. 2001; Schwartz and Vissing 2002; Piganeau et al. 2004; Zhao et al. 2004; Zsurka et al. 2005), birds (Kvist et al. 2003), fish (Magoulas and Zouros 1993; Hoarau et al. 2002; Piganeau et al. 2004; Guo et al. 2006; Ciborowski et al. 2007), reptiles (Ujvari et al. 2007), arthropods (Kondo et al. 1990; Meusel and Moritz 1993; Gantenbein et al. 2005; Arunkumar et al. 2006; Sherengul et al. 2006; Fontaine et al. 2007), molluscs (Zouros et al. 1992; Ladoukakis and Zouros 2001; Theologidis et al. 2007) and flatworms (Lunt and Hyman 1997; Piganeau et al. 2004; Armstrong et al. 2007). Although the detected cases of

either paternal leakage or recombination are assumed to be exceptions to the general rule, the increasing number of documented events clearly questions our current understanding of mitochondrial inheritance and the frequency of paternal leakage in particular.

The mechanism by which paternal leakage and subsequently heterologous recombination are prevented vary in a species-specific manner (Birky 1995; Cummins 2000). In mammals, for example, it has been shown that sperm mitochondria are ubiquitinated and subsequently degraded upon entry into the oocyte (Sutovsky et al. 1999). Other mechanisms include the dilution of paternal DNA in the oocyte, as its contribution to the overall mtDNA pool of mtDNAs in the zygote is diminutive (Ankel-Simons and Cummins 1996; Shoubridge and Wai 2007) and the exclusion of paternal mtDNA from the oocyte (Ursprung and Schabtach 1965). In addition, the presence of genetic bottlenecks during embryogenesis and oogenesis which are believed to eliminate rare mtDNA haplotypes might also tend to act against paternal transmission of mtDNA (Bergstrom and Pritchard 1998; Jansen and Burton 2004).

However, if such mechanisms fail and sperm mitochondria enter the oocyte without subsequent degradation or exclusion, paternally derived mtDNA can be inherited to offspring with the possibility of manifestation in descending progeny. Unfortunately, most studies reporting paternal leakage did not quantify the paternal contribution to the overall pool of mtDNA in samples investigated. In fact, the work of Schwartz and Vissing (2002), Sherengul *et al.* (2006), Gyllensten *et al.* (1991) and Kondo *et al.* (1990) are the only studies to estimate the paternal contribution. Studies on back-crossed and inbred mice and fruit flies found the paternal contribution to be generally between 0.01% and 0.1% of the overall mtDNA population in analyzed tissues but also documented the complete replacement of maternal with paternal mtDNA in three individuals of *Drosophila* (Kondo et al. 1990; Gyllensten et al. 1991; Sherengul et al. 2006). Schwartz and Vissing (2002)

analyzed different tissues of a man suffering mitochondrial myopathy caused by the presence of mutated paternally derived mtDNA and found the paternal mtDNA contribution to be up to 90%. Analysis of the patient's parents' mtDNA showed that both contained exclusively maternally derived mtDNA. Such findings demonstrate that if paternal leakage occurs it can be inherited as the dominant haplotype to progeny although its abundance then is originally based on just a few copies derived from sperm mitochondria. Moreover, as the enzymatic toolkit to catalyse recombination in mitochondria is widespread in the animal kingdom (Thyagarajan et al. 1996; Rokas et al. 2003) and fusion and fission have been shown to be a common feature of mitochondria (Yaffe 1999; Turnbull and Lightowlers 2001; Westermann 2002; Chan 2006), the presence of two distinct mtDNA haplotypes within single cells consequently creates the possibility for heterologous recombination to occur.

The occurrence of both paternal leakage and recombination of mtDNA in the animal kingdom has potentially substantial implications for traditional phylogenetic analysis. For example, assuming a molecular clock based on a linear rate of accumulating mutations over evolutionary time would lead to erroneous estimates if analyzed mitochondrial data sets contained sequences influenced by either event by increasing the number of potential mutations and haplotypes. Ignoring undetected recombination in genealogies can lead to underestimates for times of divergence and overestimates of the number of mutations and population size (Eyre-Walker 2000; Schierup and Hein 2000; Slate and Gemmell 2004). In the case of human evolution, for example, depending on whether recombination of mtDNA is considered or not, the estimates for our most recent common ancestor to have lived is ~171,000 years ago if recombination is ruled out (Ingman et al. 2000) and ~340,000 years ago if the applied model allowed the occurrence of recombination (Eyre-Walker 2000; Schierup and Hein 2000). As the consequences of both paternal leakage and recombination

on traditional phylogenetic analysis may be considerable, it is vital to determine at what frequency these events may occur, so that models of mtDNA evolution can be improved to estimate better evolutionary relationships and times of divergence.

Previous studies estimating the frequency of paternal leakage were greatly influenced by inbreeding and back-crossing (Kondo et al. 1990; Gyllensten et al. 1991; Shitara et al. 1998; Sherengul et al. 2006), crossing regimes that are assumed to promote paternal leakage (Kaneda et al. 1995; Sutovsky et al. 2000; Sherengul et al. 2006). Other studies detected paternal leakage but neglected to estimate how frequently this might occur (Meusel and Moritz 1993; Kvist et al. 2003; Gantenbein et al. 2005; Fontaine et al. 2007). Moreover, failures to detect paternal leakage in previous studies were probably attributable to the use of low sample sizes (Dawid and Blackler 1972; Hutchison et al. 1974; Hayashi et al. 1978; Kroon et al. 1978; Avise et al. 1979; Francisco et al. 1979; Giles et al. 1980; Reilly and Thomas 1980). A sample size of at least 300 progeny with no detected paternal mtDNA are required to correctly delimit its frequency to 1% (Milligan 1992).

Here, we have undertaken the first study of its kind to systematically estimate the frequency of paternal leakage within a species under semi-wild conditions, with potentially confounding factors such as inbreeding, back-crossing and hybridization eliminated. To achieve this, we analyzed embryos of New Zealand chinook salmon, generated through artificial fertilization, for the presence of paternal mtDNA. Chinook salmon are highly fecund and their husbandry and genetics well documented (Pennell and Barton 1996; Quinn 1996; Wilhelm et al. 2003). Single females produce up to 5,000 eggs (Pennell and Barton 1996; Kinnison 1998), providing sample sizes necessary to detect potentially rare paternal leakage and to delimit its frequency. Furthermore, sequence information of the mitochondrial genome is readily available (Wilhelm et al. 2003) and previous work in our group on this population provided us with the necessary genetic information to

unambiguously distinguish between paternally and maternally derived mtDNA (data not published).

3.3 Material and Methods

3.3.1 Marker

Previous work in our group on a wild return hatchery population of New Zealand chinook salmon revealed the presence of three main mtDNA lineages and 36 single nucleotide polymorphisms (SNPs) in the mitochondrial genes *mt-nd1* to *mt-nd5* (data not published). Each mtDNA lineage harbors at least one SNP unique for the corresponding haplogroup (diagnostic SNP). Analyzed SNPs in this study were G/A polymorphisms in the mitochondrial genes *mt-nd1* to *mt-nd3*. Nucleotide positions of these SNPs are 3957, 5842, 10650 and 10725 (hereafter single SNPs are referred to as nt3957, nt5842, nt10650, nt10725) of the mitochondrial genome (NCBI: NC_002980). All SNPs investigated were synonymous.

3.3.2 Samples

Husbandry work was carried out at the NIWA Silverstream hatchery (www.niwascience.co.nz) during the spawning season in April and May 2005. To investigate mtDNA inheritance, offspring were generated between female and male individuals from different lineages using the dry method (Huet 1970) and reared in hatching jars following standard husbandry procedures (McIntyre and Stickney 1991). Once hatched, embryos were harvested and stored in 80% ethanol at -20ºC until further

analysis. With each mtDNA lineage harboring at least one diagnostic SNP, progeny could then be analyzed for the presence of the male parent diagnostic SNP. The detection of the male parent polymorphism thus indicates the presence of paternal leakage.

3.3.3 DNA extractions

Whole genomic DNA of single embryos was extracted from tail tissue in 350 µl of extraction buffer (5% Chelex-100, 100mM NaCl, 50mM Tris [pH 8.0], 1% SDS, 10mM EDTA, 100 µg/ml RNase, 100 µg/ml Proteinase K) using a thermal shaker (55ºC, 180 rpm, 4h). After incubation, samples were centrifuged at 4,000 rpm for three minutes to precipitate cell debris and transferred into new tubes containing equal volumes of TE-Chelex-100 solution (5% chelex-100, 10mM Tris [pH 8.0], 1mM EDTA). Dilutions (1:10) were prepared and stored at -20ºC until further analysis.

3.3.4 Genotyping

Genotyping was performed using customized TaqMan®5'-nuclease assays (Applied Biosystems, Foster City, USA) in combination with the TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Foster City, USA). Experiments were carried out using a Mx3000P Q-PCR system (Stratagene, Garden Grove, USA) at reaction volumes of 10 µl containing 5-15 ng of whole genomic DNA. Q-PCR protocols consisted of an initial denaturation for three minutes at 95ºC, followed by 55 cycles with 30 seconds denaturation at 95ºC, combined annealing and elongation according to the requirements of single assays (60-63ºC) for one minute. The change in fluorescence intensity was measured

at the end of each extension step. Results were analyzed using the software MxPro, supplied with the instrument.

3.3.5 Detection limit

To evaluate the detection limit of the TaqMan®-assay for the paternal allele we prepared paternal/maternal DNA pools by adding a two-fold dilution series of paternal DNA (13 dilution steps, 4.5 ng to 0.0011 ng) to a constant remaining DNA solution of maternal DNA (9 ng), resulting in ratios of 1:2 to 1:8192. The detection limit was determined by the lowest ratio of paternal to maternal DNA for which a signal for the paternal mtDNA could be observed. Signals were considered true, if endpoint measurements were above signal threshold (T(dR)). The threshold was determined as the lowest fluorescence intensity significantly above background signal and was automatically set by the instrument.

In order to lower the detection limit, we recently showed that the sensitivity can be significantly improved by combining TaqMan®-assay with allele-specific restriction (Wolff and Gemmell 2008). Briefly, if an allele of interest (e.g. the paternal mtDNA) is outnumbered by a second allele (e.g. the maternal mtDNA), the amplification of the allele at higher frequency will dominate the PCR reaction and suppress the amplification of the allele at lower frequency and therefore prohibits its detection. We recently showed that this out-competing effect of the allele at higher frequency can be suppressed by allele-specific restriction, increasing the sensitivity of single assays 32-fold (Wolff and Gemmell 2008). This combined approach was applied to those samples that could be restricted in an allele-specific manner (paternal vs. maternal allele) at the polymorphic site. Suitable restriction enzymes, i.e. those restricting the maternal but not the paternal haplotype, were identified applying the software NEBcutter V2.0 (NEB Inc., Ipswich, USA) on the target sequence.

Physical digests of the SNP site were performed for two hours at 37ºC in a water bath using 1 unit of enzyme per 1µg DNA.

3.3.6 Calculating the frequency of paternal leakage

Maximum frequencies at which paternal leakage can be excluded were calculated according to Milligan (1992), who defined a mathematical model to determine the probability of falsely accepting strict maternal inheritance, assuming that P=0 (P = probability of paternal leakage) when in fact P>0 (Milligan 1992). According to this binominal model one can determine the maximum frequency of paternal leakage (if P=0) in correlation to the number of analyzed samples (Milligan 1992).

3.4 Results

The detection limit of standard TaqMan® assays analyzing the polymorphisms nt3957, nt10650, nt10725 and nt5842 for the paternal mtDNA were assessed by the analysis of predetermined ratios of paternal to maternal DNA. Signals indicating the presence of paternal mtDNA were reproducibly obtained for ratios between 1:2 and 1:64 (paternal to maternal DNA), whereas ratios ≤1:128 and no template controls (NTC) were below signal threshold (T(dR)), defining the detection limit for standard TaqMan® assay used in this study to be 1:64 or ~1.6% for the paternal contribution (data not shown).

The polymorphism nt3957 was identified to be suitable for allele-specific restriction prior to genotyping in order to increase the sensitivity of the TaqMan® assay. Figure 3.1 shows the amplification plots of the assay detecting the polymorphism nt3957 after

restriction on ratios ranging from 1:2 to 1:4096. Signals were reproducibly obtained for ratios between 1:2 and 1:2048, whereas fluorescence signals for ratios ≤4096 and no template control (NTC) were below signal threshold (T(dR)), restricting the detection limit for this modified TaqMan® assay to 1:2048 or ~0.05% for the paternal contribution (Figure 3.1).

Twelve independent fertilization experiments were performed and each experiment generated on average 840 progeny with 357 as the smallest and 1,677 the largest number of offspring generated in a single fertilization experiment. DNA was extracted successfully from a total of 10,082 embryos representing progeny from crossing experiments including males of all four haplogroups. According to the haplogroup and therefore to the diagnostic SNP present in the mtDNA of the male parent, 554 embryos were analyzed for the polymorphism nt10725, 3,840 for nt10650, 2,793 for nt5842 and 2,895 embryos were analyzed for the polymorphism nt3957.

Figure 3.2 shows typical results of a genotyping assay, applied on samples analyzed for the polymorphism nt3957. All samples show increasing fluorescent intensity for the maternal mtDNA whereas endpoint measurements for the paternal molecule are below signal threshold, indicating the absence of the paternal haplotype (Figure 3.2). A total of 10,082 samples have been genotyped, of which 7,188 samples were examined with a detection limit of 1:64 (samples harboring nt10725, nt10650 or nt5842) and 2,895 samples were analyzed with a detection limit of 1:2048 (samples harboring nt3957). In none of these samples paternally derived mtDNA has been detected.

Based on the results of the genotyping, we calculated the maximum frequency at which paternal leakage can be excluded to occur (Table 3.1). Table 3.1 shows a summary of all genotyping experiments and the maximum frequencies of paternal leakage in relation to sample sizes of single fertilization experiments and the power of the test. The highest

frequencies estimated in this study where 0.84% (power of 0.95) and 1.28% (power of 0.99) for a sample size of 357 and the lowest frequencies were 0.18% (power of 0.95) and 0.27% (power of 0.99) for a sample size of 1,677 (Table 3.1).

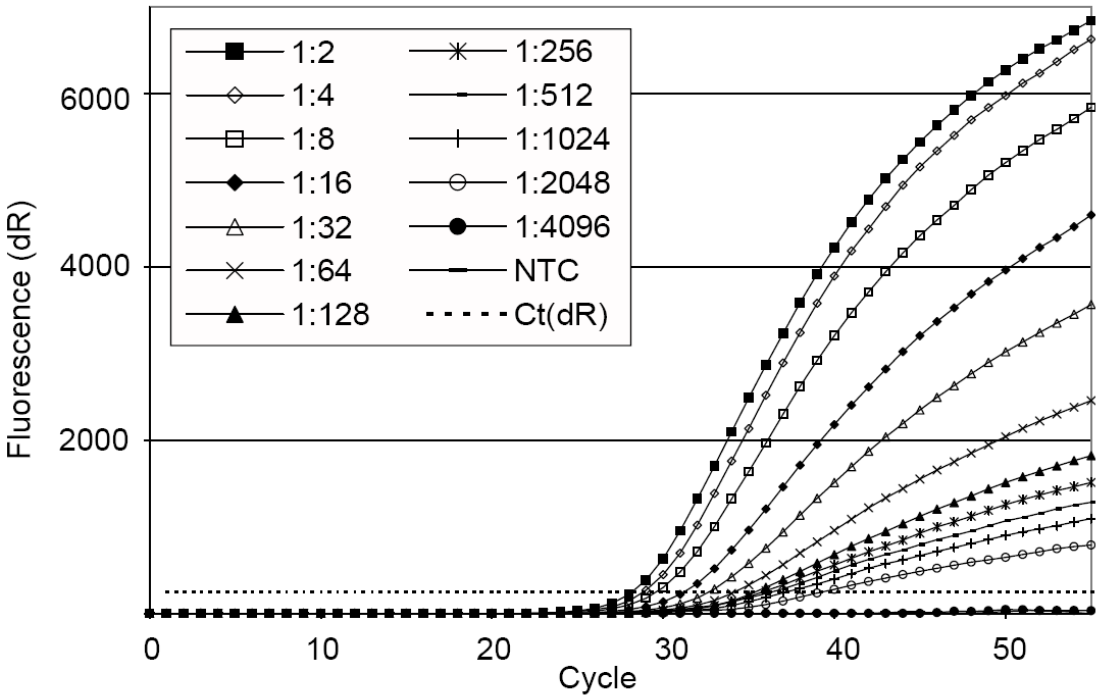

Figure 3.1: Amplification plots of the combined restriction/genotyping assay analyzing nt3957. Relative normalized fluorescence (dR) plotted against cycle number on DNA ratios of mutant to wildtype ranging from 1:2 to 1:4096 (paternal to maternal mtDNA). NTC = no template control; T(dR) = fluorescence signal threshold.

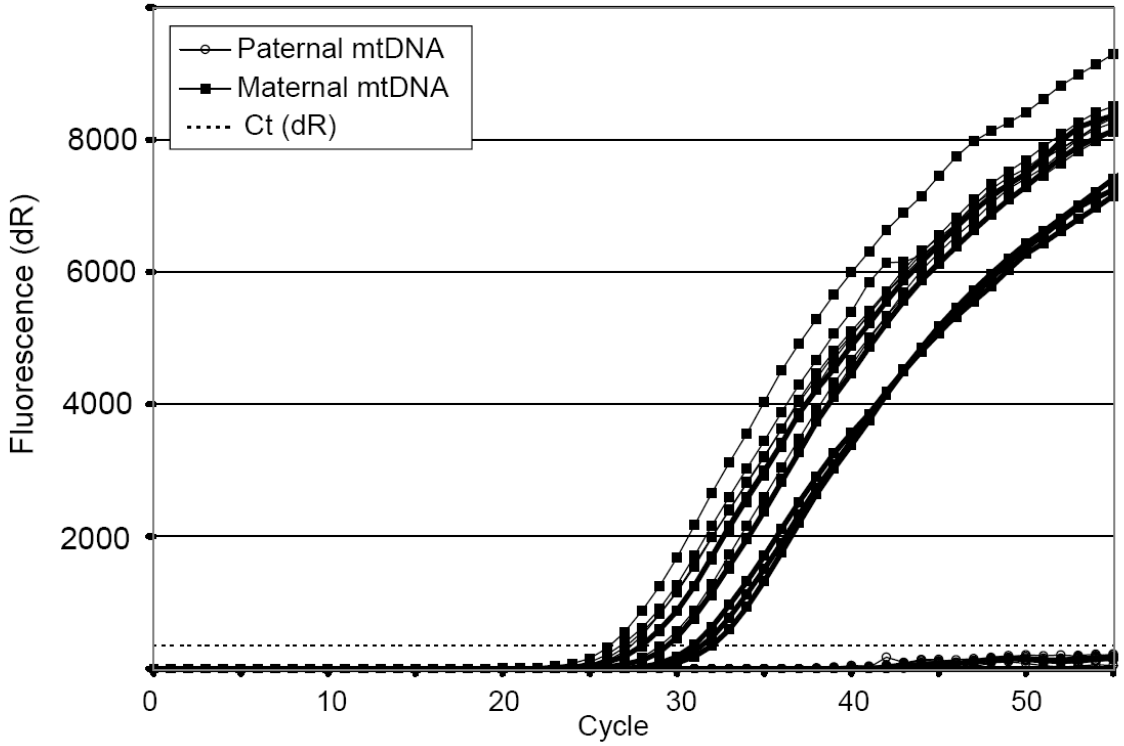

Figure 3.2: TaqMan assay applied on sample DNA. Results of the maternal fluorescent probe of the TaqMan genotyping assay applied on sample DNA harboring the polymorphism nt3957 with unknown mitochondrial load. Relative normalized fluorescence (dR) plotted against cycle number. T(dR) = fluorescence signal threshold.

Table 3.1: Maximum frequency of paternal leakage per crossing experiment. Summary of crossing experiments and upper limit of the frequency at which paternal leakage can be excluded based on sample sizes of 12 fertilization experiments. Investigated SNPs and detection limits of Q-PCR assays are indicated. Frequencies were calculated using Milligan's binominal sampling model (Milligan 1992).

Cross	SNP and (lineage)		Detection limit	Sample size	Frequency[a]	
	Female	Male			power of 0.95	power of 0.99
4	nt3957 (Z)	nt10650 (X)	1:64	523	0,0057	0,0088
10	nt10725 (Y)	nt10650 (X)	1:64	357	0,0084	0,0128
12	nt10725 (Y)	nt3957 (Z)	1:2048	660	0,0045	0,0070
13	nt5842 (Y)	nt10650 (X)	1:64	685	0,0044	0,0067
14	nt10725 (Y)	nt10650 (X)	1:64	626	0,0048	0,0073
18	nt5842 (Y)	nt10650 (X)	1:64	361	0,0083	0,0127
19	nt5842 (Y)	nt10650 (X)	1:64	1288	0,0023	0,0036
20	nt3957 (Z)	nt5842 (Y)	1:64	1677	0,0018	0,0027
24	nt5842 (Y)	nt3957 (Z)	1:2048	871	0,0034	0,0053
25	nt10650 (X)	nt10725 (Y)	1:64	554	0,0054	0,0083
26	nt5842 (Y)	nt3957 (Z)	1:2048	1364	0,0022	0,0034
28	nt10725 (Y)	nt5842 (Y)	1:64	1116	0,0027	0,0041
				10082	0,0003	0,0005

3.5 Discussion

The present study was designed to delimit the maximum frequency of paternal leakage in an intra-specific and semi-wild environment. As this frequency is expected to be potentially low, we focused on the analysis of a large sample size using an experimental set up, capable of processing this sample size and offering satisfactory sensitivity to detect minor allele contributions within single samples. Taq®Man assays are PCR-based and therefore likely to be at least as sensitive as methods used in the majority of studies revealing paternal leakage, such as PCR, PCR-RFLP and DNA sequencing (Magoulas and Zouros 1993; Schwartz and Vissing 2002; Kvist et al. 2003; Zhao et al. 2004; Gantenbein et al. 2005; Guo et al. 2006; Ciborowski et al. 2007; Ujvari et al. 2007).

We analyzed 10082 samples for the presence of paternally inherited mtDNA. None of these samples showed paternal leakage at a detectable level. According to the detection limit of both methodological approaches applied here, we could exclude paternal leakage to occur at ratios greater than 1:64 in 7,188 samples and at ratios greater than 1:2048 in 2,895 samples. The failure to detect paternally derived mtDNA in these samples does not invariably exclude the potential presence of paternal mtDNA. Firstly, the presence of paternally derived mtDNA cannot be excluded at ratios beyond the detection limits of 1:64 and 1:2048. Secondly, DNA was extracted exclusively from tissue originating from the tail of embryos, excluding the detection of paternal mtDNA (if present) in other tissues.

However, assuming the failure to detect paternal mtDNA in this study to be equivalent to the factual absence of paternal leakage, we calculated the maximum frequency at which paternal leakage can be excluded to occur in chinook salmon. The roughly 10,000 samples examined in our work originated from 12 independent fertilization experiments and progeny from different crossings were therefore treated independently.

The number of offspring per cross varied between 357 and 1,677 and the upper limit of paternal leakage accordingly between 1.28% and 0.27% (power of 0.99).

These results support the general assumption of paternal leakage being infrequent and an exception to the general rule. The occasional occurrence of paternal leakage is assumed to be due to a failure of those mechanisms to prevent paternal leakage. Twelve out of the 15 documented cases of paternal leakage were observed in hybrid zones, an environment where these mechanisms are assumed to be more relaxed and less stringent (Rokas et al. 2003). In fact, fertilization experiments in fruit flies and cattle showed that paternal leakage occurs at a significantly higher frequency in hybridization experiments compared to intraspecific crosses (Sutovsky et al. 2000; Sherengul et al. 2006). Sutovsky and co-workers (2000) demonstrated that the ubiquitination preceding the proteolytic degradation of mammalian sperm upon fertilization is not detectable in bovine hybrids whereas this process is reliable in intraspecific experiments. The absence of this process in hybrids might be due to interspecific sequence differences and therefore to differences in the amino acid sequence of proteins catalyzing this process, disabling, for example, the recognition of sperm mitochondria (Sutovsky et al. 2000).

However, the remaining three out of 15 documented cases of paternal leakage occurred either in intraspecific fertilization experiments or in natural populations (Magoulas and Zouros 1993; Schwartz and Vissing 2002; Sherengul et al. 2006), demonstrating that this phenomenon is not exclusively limited to hybrid zones. Interestingly, high frequencies of heteroplasmy, most likely caused by reoccurring paternal leakage, are also observable in natural populations of fruitflies. These incidences occur generally between individuals harboring distinct mtDNA haplotypes but also within population with only one single haplotype (Satta et al. 1988; Matsuura et al. 1991; James and Ballard 2000; Dean et al. 2003). Why mechanisms to prevent paternal leakage fail here

can only be the subject of speculation. The occurrence of paternal leakage might be simply a quantitative phenomenon, occurring accidentally in relation to time and number of fertilization events.

In summary, our results clearly indicate the rare nature of paternal leakage but as the documented cases in the literature demonstrate, the increasing disclosures of paternal leakage in recent years substantiates its occurrence beyond any doubt and it has to be considered as an ongoing part of animal mitochondrial inheritance. The occasional and infrequent occurrence of both paternal leakage and recombination of mtDNA must be considered (with particular attention paid to hybrid zones) as their presence might have significant impact on the interpretation of sequence data (Eyre-Walker 2000; Schierup and Hein 2000; Slate and Gemmell 2004).

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Chapter 4

4 Mitochondrial DNA Content of Chinook Salmon Spermatozoa*

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4.1 Abstract

Animal mitochondrial DNA (mtDNA) is inherited predominantly maternally. Various mechanisms to avoid the transmission of paternal mtDNA to offspring have been proposed including the dilution of paternal mtDNA by maternal mtDNA in the zygote. The effectiveness of dilution as a barrier will be determined by the number of mtDNA molecules contributed by each parental gamete and is expected to be highly variable among different taxa due to interspecific differences in mating systems and gamete investment. Estimates of this ratio are currently limited to few mammalian species and data from other taxa are therefore needed to understand better the mechanisms of mitochondrial inheritance. The present study estimates the mtDNA content in salmon sperm at 5.73 ± 2.28, similar to that observed in other vertebrates. Although highly divergent, it appears that the mitochondrial DNA content may be conserved within vertebrate taxa, indicating the reduction of mtDNA being a key factor of spermatogenesis to ensure mitochondrial functionality on the one hand and to avoid paternal leakage at a significant or detectable level on the other hand. We employ quantitative real-time PCR (Q-PCR) and demonstrate the accuracy and high reproducibility of our experiments. Furthermore, we compare and evaluate two standard approaches used for the quantification of genes, Q-PCR and blotting methods, in regard to their utility to accurately quantify mitochondrial genes.

4.2 Introduction

Animal mitochondrial DNA (mtDNA) is a circular double-stranded molecule normally varying between 15 and 20 kb in length. It generally encodes 22 transfer RNAs, two ribosomal RNAs and 13 subunits of the oxidative phosphorylation system and is highly conserved in animals (Wolstenholme and Clary 1985; Kocher et al. 1989; Shadel and Clayton 1997; Saraste 1999). The laws for the inheritance of the mitochondrial genome differ from those revealed for nuclear genes. Intergenerational transmission follows non-Mendelian rules and replication of the mitochondrial genome occurs independently from the cell cycle (Birky 2001), with mtDNA typically assumed to be inherited exclusively through the maternal line. Paternal transmission of mtDNA (hereafter: paternal leakage) and recombination of mtDNA are considered to be absent, or at most, rare occurrences in animals (Brown et al. 1979; Birky 1995; Ballard and Dean 2001; Rokas et al. 2003).

Why mtDNA follows a maternal mode of transmission is the subject of speculation. A favored hypothesis follows from observations that mutational damage caused by free radicals can reduce mitochondrial activity (Rand 1994; Lenaz 1998). Therefore, in systems revealing oogamy, where non-motile eggs are fertilized by motile sperm cells, the inheritance of maternal mtDNA might be favored over paternal mtDNA which potentially accumulates mutations during the time of oxidative activity required to reach the egg, whereas the oocyte`s mtDNA should be still in a relatively pristine state (Allen 1996). An alternative explanation is that the exclusion of paternal mitochondria may reduce the influence of ‘selfish’ mitochondria. If mitochondria were inherited from both parents, zygotes would contain two kinds of mitochondria (paternal and maternal) with genetically distinct mtDNA haplotypes. If one haploptype increases its own rate of replication but is

associated, for example, with reduced metabolic activity, its frequency will nonetheless increase and potentially decrease both individual and population fitness (Hastings 1992; Hurst 1996). As mentioned, biparental inheritance of mtDNA molecules could lead to such a scenario, but as this is maladaptive from the perspective of the nuclear genome, long term persistence of two mitochondrial types in the same cell should be rare, with uniparental inheritance highly favored.

Recent studies however challenge the dogma surrounding mitochondrial inheritance with accumulating evidence for paternal leakage in a wide range of animal species (summarized in Fontaine et al. 2007). The mechanisms by which paternal leakage is prevented vary in a species-specific manner (Birky 1995). These mechanisms include active ubiquitination and subsequent destruction of sperm mitochondria (Sutovsky et al. 2000; Thompson et al. 2003), the exclusion of sperm mitochondria from the oocyte (Ursprung and Schabtach 1965) or the presence of a genetic bottleneck eliminating rare haplotypes, such as paternally transmitted mtDNA (Bergstrom and Pritchard 1998; Jansen and de Boer 1998, Cao et al. 2007). However, one mechanism seems to apply to most animal species: Paternal mtDNA is assumed to be vastly outnumbered by maternal mtDNA upon fertilization (Ankel-Simons and Cummins 1996; Rokas et al. 2003).

To describe this dilution effect and the ratio of paternal to maternal mtDNA in zygotes, the quantification of mtDNA per gamete has become the subject of interest. The few reports available to date document widely varying estimates and are limited to few mammalian species. In four separate studies, mtDNA content of human sperm was found to be 1.4, 6.8, 1000 and 1500 molecules per sperm (Manfredi et al. 1997; Diez-Sanchez et al. 2003; May-Panloup et al. 2003; Amaral et al. 2007) and the mtDNA content per mouse sperm was estimated at 10 and 75 molecules (Hecht et al. 1984; Shitara et al. 2000). Mammalian oocytes instead contain between $0.5x10^5$ and $1.5x10^6$ copies of the

mitochondrial genome (Shoubridge and Wai 2007), leading to ratios of roughly $1:10^3$ to $1:10^6$ paternal to maternal mtDNA molecules in fertilized zygotes.

The present study estimates mtDNA content in salmon sperm, the first non-mammalian vertebrate to be examined, to work towards the description of the maternal/paternal mtDNA ratio in a fertilization system completely different to that of mammals (Jalabert 2005). The majority of teleosts release gametes during spawning into an aqueous environment with fertilization occurring externally (Jalabert 2005). As an adaptation to this environment teleost spermatozoa have retained a simple structure, with a rounded head lacking an acrosome and a collar-like midpiece containing a few mitochondria (Nagahama 1983; Gusmao-Pompiani et al. 2005). Sperm of internal fertilizers, such as mammals, have both an elongated head with acrosome and midpiece (Bahr and Engler 1970; Bedford and Hoskins 1990; Gage 1998). Moreover, mammalian sperm contain as many as 75 to 100 mitochondria which can be merged or fused to assemble extensive mitochondrial structures (Bahr and Engler 1970; Bedford and Hoskins 1990; Gage 1998). The data available for teleosts indicate the number of mitochondria only up to ten per sperm (Lahnsteiner 2003; Quagio-Grassiotto et al. 2003; Gusmao-Pompiani et al. 2005). The limited number of mitochondria in teleost sperm can be irregularly shaped and single mitochondria can fuse to either build a ring-like structure around the axoneme or to merge to one single large mitochondrion (Gusmao-Pompiani et al. 2005; Quagio-Grassiotto and Oliveira 2008). The period of sperm motility in externally fertilizing fish is generally short with time spans of two minutes and less (Stoss 1983; Burness et al. 2005), whereas mammalian sperm are generally both fertile and motile for one day and longer (Gomendio et al. 1998). Considering differences in energy demand, adaptations to the environment and spermatozoan morphometry between mammals and teleosts, the mtDNA content of spermatozoa and consequently the mtDNA ratio might vary considerably. As a

first step towards the description of the mtDNA ratio in teleost zygotes, we quantified the mtDNA of New Zealand chinook salmon (*Oncorhynchus tshawhytscha*) spermatozoa. We employed Q-PCR and demonstrate the accuracy and high reproducibility of this approach and further discuss the general applicability of Q-PCR in comparison to blotting methods to quantify mitochondrial genes.

4.3 Materials and Methods

4.3.1 Sperm samples

Samples were collected during the spawning season in April and May 2005 in collaboration with the NIWA Silverstream Hatchery, located north of Christchurch on the Kaiapoi River. One to five ml of milt was squeezed manually from 14 males, following standard husbandry procedures (Billard and Jensen 1996). All individuals were semi-wild returns, i.e. fish reared at the hatchery, released into the wild, and that had return for spawning to the hatchery.

4.3.2 Preparation of DNA

Prior to extraction, sperm samples were diluted 1:6 in TE buffer (pH 8.0) and cells were disrupted using an ultrasonic probe (VC 750, Sonics, Newtown, USA) at 20 kHz for one minute. DNA was extracted from 50 µl of disrupted sperm cells using 450 µl of extraction buffer, containing 5% Chelex-100 (Sigma-Aldrich, St Louis, USA) in TE (pH 8.0) and Proteinase K (Roche, Indianapolis, USA) at a final concentration of 0.4 µg/µl. Samples were incubated using a thermal shaker for 5 hours at 55 ºC with shaking intervals of 5 sec

at 1400 rpm every 10 min. DNA extracts were not purified to avoid significant loss of template DNA during the purification process. After incubation, samples were centrifuged, diluted 1:40 in TE (pH 8) and stored at -20ºC. To exclude the presence of inhibitory factors in DNA extracts and to control for satisfactory PCR efficiencies, standard curves from two-fold dilution series of sample DNA were derived.

The extraction method used here was adapted from those used on mammalian sperm. Although morphologically distinct, differences in extraction efficiency between mammalian and salmon sperm using this method were considered non-significant but could not be excluded. Therefore, additional extrartion trials with a range of detergents (such as Tween-20, Nonidet P40, DTT, etc.) and with different duration of extraction (i.e., 1, 2, 3, …, 9, 10 hours) were conducted to evaluate the efficiency of the original protocol. Gene copy numbers were determined during these trials and the optimal protocol defined. Additional detergents did not reasult in increasing gene copy number whereas an increase in copy number could be observed until three hours during the time trial. Consquently the duration of extraction was set at five hours. To further support cell disruption, sample were sonicated.

4.3.3 Quantification of the mitochondrial genome (Q-PCR)

To quantify the number of mtDNA molecules, we used a 314-bp fragment of the mitochondrial *mt-nd1* gene as external standard. *mt-nd1* encodes a subunit of the NADH dehydrogenase of the respiratory complex I in mitochondria (Nosek and Fukuhara 1994). The fragment was amplified using the forward primer ND1aF (5`-GGTAATTGCGAGAGGCCTAA-3`) and the reverse primer ND1R1 (5`-GTAAGGGCAAGTATGGGTGT-3`). PCR reactions were carried out with 25 ng of total

DNA in a 25 µl volume, 10 pmol of each primer, 2 nmol of each dNTP and 0.5 U of BioTaq™ (Bioline, London, UK). PCR amplification consisted of 2 min initial denaturation and 30 cycles of denaturation at 94ºC for 15 sec, annealing at 57ºC for 20 sec and elongation at 72ºC for 20 sec. The PCR product was cloned using pGEM-T Easy PCR cloning kit (Promega, Madison, USA) and the recombinant plasmid was purified using PureLink™ Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, USA). Clones were analyzed in-house on a capillary ABI3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, USA). The purity of the recombinant plasmid was checked by spectrophotometry using a ND-1000 NanoDrop (NanoDrop Tech., Wilmington, USA) and the absence of residual genomic DNA by gel electrophoresis. The recombinant plasmid was 3,329 bp in length (314 bp insert and 3,015 bp vector pGEM-T Easy) and 1µg of DNA was estimated to contain 2.79×10^{11} molecules (http://molbiol.edu.ru/eng/scripts/h01_07.html). Ten-fold serial dilutions with known copy numbers were prepared, ranging from 10^2 copies/µl to 10^7 copies/µl. DNA solutions were aliquoted and stored at -20ºC.

Samples were analyzed using a Mx3000P Q-PCR system (Stratagene, Garden Grove, USA) in combination with the Fast Start SYBR Green Master mix (Roche, Indianapolis, USA). Sample and reference DNA were analyzed in 20 µl reaction volumes, containing 1x Fast Start SYBR Green Master mix, 1 µl DNA and 10 pmol of each primer (ND1aF and ND1R1). Q-PCR amplifications consisted of an initial denaturation for 10 min at 95ºC, followed by 40 cycles with 20 sec denaturation at 95ºC, annealing for 20 sec at 57ºC and elongation at 72ºC for 20 sec. The change in SYBR green fluorescence intensity was measured at the end of each extension step. After 40 cycles a melting curve analysis was performed to screen the reaction for non-specific amplification. For each experiment a standard curve was generated, using the 10-fold serial dilutions of the

recombinant plasmid (10^2 to 10^7 copies), to quantify the number of mtDNA molecules in samples investigated. All samples, the external standard and no template controls were tested in triplicate and results were analyzed using the software MxPro, supplied with the instrument.

Although no nuclear encoded mitochondrial pseudogenes (numts) have to date been reported in fish (Bensasson et al. 2001; Venkatesh et al. 2006), we performed a BLAST search of the *mt-nd1* sequence against several salmonid databases to exclude the possible amplification of such genes. BLAST searches were performed against rainbow trout (*O. mykiss*) in particular (http://web.uvic.ca/cbr/grasp/) and against salmonid databases in general (www.salmongenome.no/cgi-bin/sgp.cgi, www.ncbi.nlm.nih.gov/blast/).

4.3.4 Estimation of the number of sperm by quantification of the single-copy gene *rag1* (Q-PCR)

To determine the number of sperm per ml milt, we quantified a sequence of the V(D)J recombination activating gene (*rag1*), a single-copy gene, encoded in the nuclear genome (Schatz et al. 1989). The insert for the recombinant plasmid was a 166-bp fragment, amplified with the forward primer RagF (5`-AGATGGTGCGAGAAATGGAG-3`) and the reverse primer RagR (5`-TGAGGATTGGTCCTCCAAAG-3), derived from sequence information of rainbow trout (*Oncorhynchus mykiss*) (NCBI: U15663; Hansen and Kaattari 1995). The actual quantification however, was carried out using the forward primer RagF2 (5`-TGAGAAGATGGTGCGAGAAA-3`) and the reverse primer RagR3 (5`-CGGGTGACAGAGTGGAGAGT-3`), spanning a sequence of 120 bp. The change of primers to amplify this smaller fragment was made due to low PCR efficiencies (80-85%, data not shown) obtained with primers RagF/RagR.

PCR reactions to produce the insert for the external standard were carried out as stated above for the quantification of mtDNA using 100 ng of template DNA per reaction. The fragment was cloned using GeneJet^{TM} PCR cloning kit (Fermentas, Hanover, USA) and the recombinant plasmid was 3,294 bp in length (166 bp insert and 3,128 bp vector pJET1). Purification and quality testing was performed as described above for mtDNA. One µg of DNA was estimated to contain 2.82 x 10^{11} molecules (http://molbiol.edu.ru/eng/scripts/h01_07.html). Ten-fold serial dilutions with known copy numbers were prepared, aliquoted and stored at -20ºC. Q-PCR experiments were performed and analyzed as described above for the quantification of the *mt-nd1* sequence with a change in annealing temperature to 58ºC. Again, for all experiments standard curves were generated using the dilution series of the recombinant plasmid (10^2 to 10^7 copies/µl). All samples were measured in triplicate and results were analyzed using the software MxPro, supplied with the instrument.

The calculation of mtDNAs per ml milt and sperm per ml milt were made with consideration of all dilutions of both the milt sample and the DNA extract. These include dilution steps for cell disruption (6x), DNA extraction (10x), Q-PCR (40x) and calculations per ml (1000x). The number of mtDNAs per single sperm was then determined by the ratio of mtDNAs per ml milt and the number of *rag1* copies per ml milt, taking into account that chinook salmon are tetraploid (Allendorf and Thorgaard 1984). This means that single gametes have two sets of chromosomes and therefore two copies of *rag1*. Consequently, two copies of *rag1* were assigned to one sperm.

The applicability of the *rag1* screen for the estimation of sperm present in our DNA samples was tested against sperm counts using a hemocytometer (Tvedt et al. 2001; Eshre Monographs 2002). Sperm numbers were estimated five times using both a hemocytometer and Q-PCR and the mean values of both approaches were compared using

the Mann-Whitney test. The use of a hemocytometer is generally well-established and highly suitable to conduct sperm counts. However, if a sperm analyzer, such as CASA, is available, the use of such instrument would be preferable.

4.4 Results

4.4.1 Number of mtDNA molecules per ml milt

To determine the number of mtDNAs per sperm, we first quantified the mitochondrial gene *mt-nd1* in our samples. Amplifications of the ten-fold dilution series of the external standard (Figure 4.1) and the two-fold dilution series of sample DNA (data not shown) were carried out successfully. The efficiencies were 99.2% (Figure 4.2) and 99.1% (data not shown) for the reference DNA and the sample DNA, respectively. Furthermore, standard curves derived from both dilution series showed highly similar slopes with -3.341 for the reference DNA (Figure 4.2) and -3.344 for the sample DNA (data not shown). Melting curve analysis demonstrated that Q-PCR experiments resulted in the amplification of one single sequence and a BLAST search for mitochondrial pseudogenes did not detect any similar sequences other than mitochondrial *mt-nd1* sequences (data not shown), excluding the possibility of unspecific amplification. This Q-PCR assay was applied to a total of 14 individuals. The mean number of mtDNAs within the analyzed samples was $2.82 \times 10^{10} \pm 9.58 \times 10^{8}$ molecules per ml milt (Table 4.1).

4.4.2 Number of sperm per ml milt

After we determined the number of mtDNA molecules present in sperm extracts, we assigned these molecules to the estimated number of sperm. To achieve this, we quantified the single-copy gene *rag1*. To confirm the utility of this Q-PCR screen and to allow for accurate estimates, PCR efficiencies for sample DNA and external standard must reveal high degrees of similarity. To determine the PCR efficiency for sample DNA, a two-fold dilution series of sample DNA was prepared and investigated. Amplifications efficiencies were 98.8% and 98.5%, slopes -3.352 and -3.358 for the reference DNA and sample DNA, respectively (data not shown). This second screen revealed an average of $1.02 \times 10^{10} \pm 5.31 \times 10^{8}$ copies of *rag1* per ml milt (Table 4.1).

Results from sperm counts using either a hemocytometer or Q-PCR were highly similar and the application of the Mann-Whitney test on these data demonstrated that documented differences were not significant (Table 4.2).

4.4.3 Number of mtDNA molecules per sperm

Finally, estimates for the number of mtDNAs per sperm were determined by correcting the mtDNA copy numbers per ml by the number of *rag1* copy number per ml (Table 4.3). Chinook salmon are tetraploid, so two copies of *rag1* were assigned to one sperm. Accordingly, the lowest copy number detected in this study was 2.77 mtDNAs per sperm and the highest 9.42 mtDNAs per sperm with an overall average of 5.73 ± 2.28 molecules per sperm (Table 4.3).

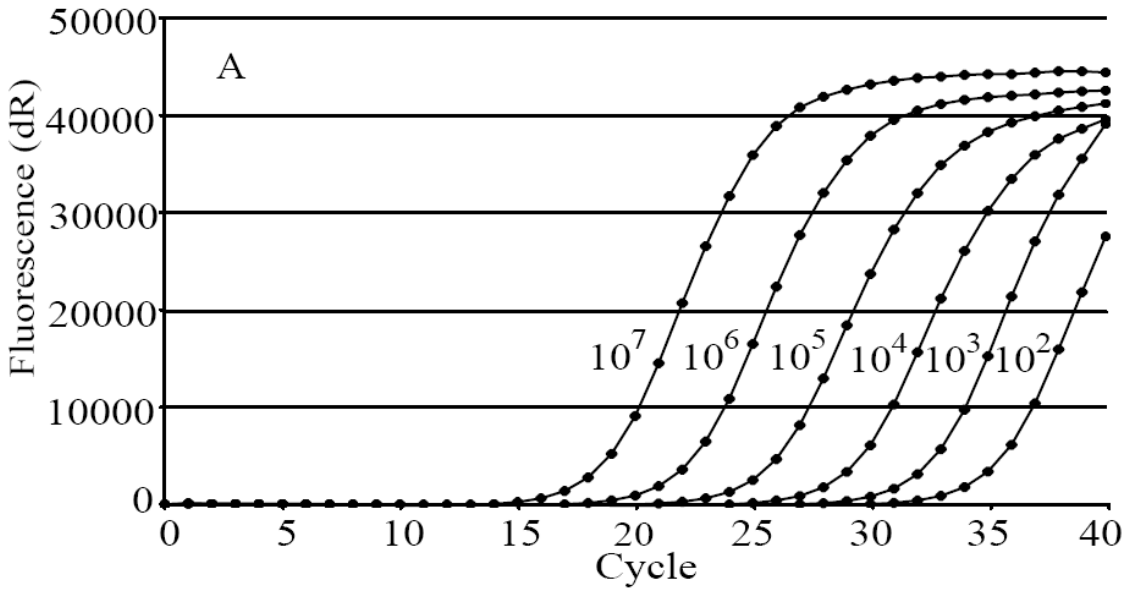

Figure 4.1: Amplification plots of the external standard. Amplification plots of ten-fold dilution series of the recombinant plasmid (*mt-nd1* insert) for the quantification of mtDNAs (10^2 to 10^7 copies). Normalized flourescence of PCR amplifications plotted against cycle number.

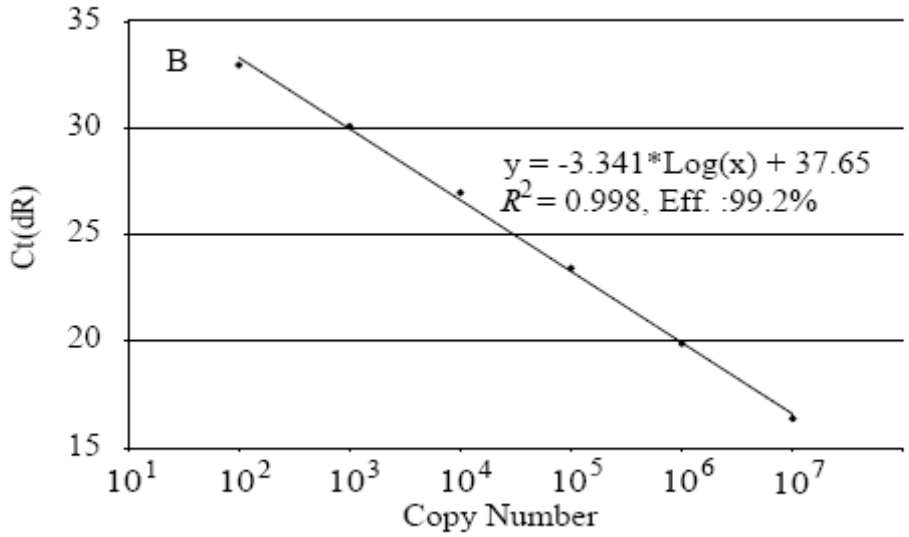

Figure 4.2: Standard curve for the quantification of the mitochondrial genome. Threshold cycle plotted against copy number derived from amplifications of the external standard. PCR efficiency: 99.2%, RSq: 1.000, slope: - 3.341 (Y= -3.341 *LOG(X) + 37.65).

Table 4.1: Number of mtDNA molecules and copy number of *rag1* per ml milt among 14 individuals of New Zealand chinook salmon.

Individual	*rag1* copies per ml milt [x10^{10}]	Standard-deviation [x10^{8}]	CV[1](%)	mtDNAs per ml milt [x10^{10}]	Standard-deviation [x10^{8}]	CV[1](%)
05-393	0.90	1.09	1.21	1.45	0.98	0.67
05-397	0.78	3.29	4.22	2.52	20.20	8.03
05-400	0.76	2.47	3.25	3.09	14.80	4.78
05-405	0.92	3.02	3.27	2.11	2.77	1.31
05-415	0.80	1.75	2.18	2.38	2.84	1.19
05-421	0.83	3.97	4.77	3.66	12.70	3.45
05-423	0.78	3.06	3.92	3.33	16.40	4.91
05-430	0.94	7.21	7.71	1.47	5.42	3.70
05-435	0.66	3.34	5.04	1.60	6.28	3.92
05-436	1.09	13.60	12.47	3.47	9.88	2.85
05-438	1.57	3.97	2.53	2.93	13.20	4.51
05-439	1.35	9.54	7.08	6.35	20.70	3.26
05-440	1.39	12.10	8.69	2.99	4.78	1.60
05-442	1.53	5.91	3.87	2.12	3.24	1.53
Mean	1.02	5.31	5.02	2.82	9.58	3.27

[1]coefficient of variance

Table 4.2: Comparison of Q-PCR and hemocytometer to determine sperm density. Results for three sperm samples with 5 replicates each and subsequent analysis using the Mann-Whitney test (sperm densities $x10^9$).

Sample	1		2		3	
	HC[1]	Q-PCR	HC[1]	Q-PCR	HC[1]	Q-PCR
Replicates	4.21	4.93	5.95	5.27	5.25	5.01
	4.55	4.92	5.16	5.85	4.96	5.11
	5.35	4.97	6.15	5.48	5.95	4.82
	4.86	4.77	5.65	5.83	4.23	5.02
	4.15	4.57	4.97	5.22	5.25	5.00
Mean	4.63	4.83	5.58	5.53	5.13	4.99
Stdv[2]	0.40	0.16	0.50	0.30	0.62	0.11
CV[3][%]	10.72	3.39	9.04	5.37	12.13	2.13
M-W[4]	U:18; P_1:0.15;P_2:0.3		U:12; P_1:0.5;P_2:1.0		U:18; P_1:0.26;P_2:0.53	

[1]Hemocytometer; [2]Standarddeviation; [3]Coefficient of variance; [4]Mann-Whitney test; P_1: P (one-tailed); P_2: P (two-tailed)

Table 4.3: Number of MtDNAs per Sperm**.** Mean number of mtDNA copies per sperm among 14 individuals of New Zealand chinook salmon.

Individual (tag-no.)	Mean of mtDNAs per ml milt [$x10^{10}$]	Mean of sperm per ml milt [$x10^{9}$]	Number of mtDNAs per sperm
05-393	1.45	4.48	3.24
05-397	2.52	3.90	6.45
05-400	3.09	3.80	8.13
05-405	2.11	4.61	4.59
05-415	2.38	4.02	5.92
05-421	3.66	4.16	8.81
05-423	3.33	3.90	8.53
05-430	1.47	4.68	3.13
05-435	1.60	3.31	4.84
05-436	3.47	5.46	6.34
05-438	2.93	7.85	3.73
05-439	6.35	6.73	9.42
05-440	2.99	6.95	4.30
05-442	2.12	7.64	2.77
Mean	2.82	5.11	5.73 ± 2.28

4.5 Discussion

This study revealed an average of 5.73 ± 2.28 mtDNA copies per sperm among 14 individuals of New Zealand chinook salmon using Q-PCR. The quantification of genes is generally achieved by Q-PCR or blotting methods, such as Southern or Slot blotting. Both methodological approaches have been applied to estimate the mtDNA content of spermatozoa in human and mouse (Hecht et al. 1984; Manfredi et al. 1997; Shitara et al. 2000; Diez-Sanchez et al. 2003; May-Panloup et al. 2003; Amaral et al. 2007). However, estimates presented in these studies vary significantly with blotting methods usually documenting estimates which are up to three orders of magnitude higher than those inferred by Q-PCR (Hecht et al. 1984; Manfredi et al. 1997; Shitara et al. 2000; Diez-Sanchez et al. 2003; May-Panloup et al. 2003; Amaral et al. 2007). Two major concerns have been raised to explain this discrepancy: Firstly, it has been suggested that the presence of considerable amounts of genomic DNA in Q-PCR experiments may act inhibitory on the amplification of the target sequence (Diez-Sanchez et al. 2003). Inhibition of amplification typically results in less PCR product and therefore an underestimation of mtDNA content. However, in the present study we demonstrate that this effect can be easily avoided by diluting the sample DNA. By creating serial dilutions of sample DNA and performing Q-PCR experiments on these dilutions, the dilution at which the reaction is no longer inhibited can be quickly determined. PCR reactions of external standards and of two-fold serial dilutions (1:40-1:320) of sample DNA in this study revealed efficiencies of 99.2% vs. 99.1% for *mt-nd1* and 98.8% and 98.5% for *rag1*, respectively. The high degree of similarity in amplification efficiency therefore confirmed the absence of any inhibitory influence of nuclear DNA.

The second explanation put forward is that the influence of nuclear-encoded mitochondrial pseudogenes (numts) on the quantification of mtDNAs may lead to

erroneous results (May-Panloup et al. 2003). In the human genome, as many as 300 numts have been detected (Mourier et al. 2001). These numts often bare a remarkable homology to the original mitochondrial genes, with the majority of numts (78%) differing only by length polymorphisms caused by small deletions or insertions (Tourmen et al. 2002; Woischnik and Moraes 2002). Considering the low level of difference between numts and genuine mtDNA sequences, it has been suggested that the detection of high numbers of mtDNAs per sperm via blotting methods, may result from cross-hybridization of DNA probes to numts (May-Panloup et al. 2003). In Q-PCR, this potential error source can be excluded by performing a melting curve analysis. Such analysis reveals the presence of additional PCR products other than the target sequence, based on differences in length and base composition. In our study, we performed such analysis after each Q-PCR experiment and could therefore verify the successful amplification of our target sequence and exclude the accidental amplification of any additional sequences. Moreover, although no numts have been detected in a teleost (Venkatesh et al. 2006), we further excluded the possibility that we may be amplifying nuclear copies of our target *mt-nd1* sequence by BLASTing this sequence against Genbank and several salmonid databases. No sequences were recovered from these searches other than known mitochondrial *mt-nd1* sequences of various salmonid fish.

Considering that potential cross-hybridization with numts cannot be excluded using blotting methods and that the influence of inhibitory factors can be easily avoided by diluting sample DNA, we suggest that Q-PCR is the favored method for the quantification of spermatozoal mtDNA content. This method also has the advantage of high efficiencies, reproducibility and low standard deviations. The coefficients of variation, a measure of standard deviation, for the estimation of sperm density in our experiments were 5.02% and 3.27%. Furthermore, a comparison of our Q-PCR screen against sperm counts using a

hemocytometer confirmed Q-PCR as suitable for the determination of sperm densities and therefore for the quantification of single genes (Table 2).

Applying our Q-PCR assay, we determined the mtDNA content among 14 individual chinook salmon to be on average 5.73 ± 2.28 mtDNA genomes per sperm. This estimate is similar to those revealed for mice and human, using a comparable Q-PCR approach (Shitara et al. 2000; May-Panloup et al. 2003; Amaral et al. 2007). These studies estimated the mtDNA copy number to be approximately 10 for mouse spermatozoa and 1.4 and 6.8 for human spermatozoa, respectively (Shitara et al. 2000; May-Panloup et al. 2003; Amaral et al. 2007). Considering differences in spermatozoal morphology, mitochondrial complexity and life traits between the two systems, the similarity in mtDNA content of teleost and mammalian sperm is unexpected (Stoss 1983; Gomendio et al. 1998; Lahnsteiner 1998). In contrast to teleost spermatozoa, mammalian spermatozoa can be active for several days (Gomendio et al. 1998). We anticipated the difference in energy demand according to the duration of motility of both systems to be reflected in the mtDNA load of single gametes. This assumption is in concordance with the observation that mammalian sperm contain 75 to 100 mitochondria (Bahr and Engler 1970; Ho and Wey 2007) whereas teleost sperm merely contain up to 10 mitochondria (Lahnsteiner 2003; Gusmao-Pompiani et al. 2005). Given that each mitochondrion is assumed to contain at least one copy of the mitochondrial genome (Cummins 1998), the estimated 10 copies of mtDNA per sperm in mouse (Shitara et al. 2000) and 1.4 and 6.8 in human (May-Panloup et al. 2003; Amaral et al. 2007) appear to be unexpectedly low, whereas the estimated 5.73 ± 2.28 mtDNA molecules per teleost sperm with an observed maximum of 10 mitochondria per sperm appear to be within our expectations. What remains to be elucidated is how the apparent discrepancy between mtDNA content and number of mitochondria per mammalian sperm can be explained.

Firstly, mitochondria have been repeatedly reported to fuse during spermatogenesis to form complex mitochondrial structures such as the mitochondrial sheath where single mitochondria can either merge or be fused end-to-end (Sawada et al. 1975; Gould and Martin 1977; Hales and Fuller 1997; Plön and Bernard 2006). Only a limited number of mtDNA molecules are probably required to maintain mitochondrial functionality, thus it is conceivable that the overall mtDNA content of merged mitochondria decreases post-fusion (or may already have decreased pre-fusion) through active degradation of mtDNA with several joint mitochondria sharing the same pool of mtDNA. Secondly, considering the limited lifespan of spermatozoa, it is arguable if mtDNA is required at all to maintain mitochondrial functionality during this period of time. Instead, it is conceivable that the presence of stable mtDNA transcripts, which are translated into subunits of the respiratory chain, may be sufficient to allow for mitochondrial functionality until fertilization. Both arguments can potentially serve to explain the low mtDNA in mouse and human and are in concordance with the evident mtDNA reduction of mtDNA during spermatogenesis in mammals (Rantanen et al. 2001; Nishimura et al. 2006).

However, contrary to our expectation that we might find a correlation between mtDNA content, energy demand and mitochondrial complexity, it appears that despite the high divergence between teleosts and mammals, both in terms of life-history and evolutionary distance, that the mtDNA content of sperm is conserved within these vertebrate taxa. This may indicate that the reduction of mtDNA during spermatogenesis is a key part to ensure mitochondrial functionality on the one hand and to prohibit paternal leakage on the other hand. Additional studies across species are desirable to further confirm this hypothesis.

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Chapter 5

5 Lost in the Zygote: The Dilution of Paternal mtDNA Upon Fertilization*

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5.1 Abstract

The mechanisms by which paternal inheritance of mtDNA (paternal leakage) and subsequently recombination of mtDNA are prevented vary in species-specific manner with one mechanism in common: paternal derived mtDNA is assumed to be vastly outnumbered by maternal mtDNA in the zygote. To date, this dilution effect has only been described for two mammalian species, human and mouse. Here, we estimate the mtDNA content of salmon oocytes to evaluate the dilution effect operating in another vertebrate; the first such study outside a mammalian system. Employing real-time PCR, we determined the mtDNA content of salmon oocytes to be $3.15x10^9 \pm 9.98x10^8$ and recently, we determined the mtDNA content of chinook salmon sperm to be 5.73 ± 2.28 per gamete. Accordingly, the ratio of paternal to maternal mtDNA if paternal leakage occurs is estimated to be $1:7.35x10^8 \pm 4.67x10^8$. This contribution of paternal mtDNA to the overall mtDNA pool in salmon zygotes is 3 to 5 orders of magnitude smaller than those revealed for the mammalian system, strongly suggesting that paternal inheritance of mtDNA per offspring will be much less likely in this system than in mammals.

5.2 Introduction

Animal mitochondrial DNA (mtDNA) is the marker of choice for a wide range of applications spanning phylogeography, phylogenetics and population genetics (Brown et al. 1979; Avise et al. 1987). Central to its success are several key characteristics, such as its high copy number, small size (generally 15 to 20 kb), higher mutation rate (compared to nuclear DNA) and clonal inheritance. The presumption of strict maternal transmission, and the subsequent lack of heterologous recombination, enabled the investigation of complex genetic traits without the aggravating complexities of biparental inheritance and recombinant genetic information (Brown et al. 1979; Bromham and Penny 2003; Slate and Gemmell 2004).

Although it has been argued that paternal inheritance should be suppressed to avoid potentially lethal genome conflict (Hurst 1996), there is evidence of rare but potentially significant paternal leakage and recombination of mtDNA in a wide range of animal species. Both events have been documented to occur in mammals (Gyllensten et al. 1991; Schwartz and Vissing 2002; Ladoukakis and Eyre-Walker 2004; Zhao et al. 2004), birds (Kvist et al. 2003), fish (Magoulas and Zouros 1993; Guo et al. 2006; Ciborowski et al. 2007), mollusks (Zouros et al. 1992), reptiles (Ujvari et al. 2007), arthropods (Meusel and Moritz 1993; Gantenbein et al. 2005; Arunkumar et al. 2006; Sherengul et al. 2006; Fontaine et al. 2007) and flatworms (Lunt and Hyman 1997). Although not the general rule, the increasing number of documented cases for these events clearly questions our current knowledge of mitochondrial inheritance, and in particular the validity of our assumptions in a general sense, as well as at a taxon specific level.

The mechanisms by which paternal leakage and recombination of mtDNA are prevented vary in a species-specific manner (Birky 1995), however there is one mechanism

in common: the many-fold dilution of paternal mtDNA in the zygote. If paternal mitochondria, and therefore the mtDNA they contain, enter the egg without subsequent degradation, the paternally derived mtDNA is assumed to be vastly outnumbered by maternal mtDNA (Birky 1995; Ankel-Simons and Cummins 1996).

The ratio of paternal to maternal DNA in the zygote will be determined by the mtDNA content of the parental gametes. However, this ratio does not necessarily reflect the extent of paternal leakage in the developing embryo. Although the subject of controversy, it is generally accepted that the amount of mtDNA does not change during early embryogenesis up to the blastocyst and early egg cylinder stage in mice (Piko and Matsumoto 1976; Ebert et al. 1988; McConnell and Petrie 2004). At this stage, the embryo has already undergone numerous cell divisions and mitochondria that were present in the zygote are assumed to be apportioned to these cells in a random fashion. During these early cell divisions, cell lineages such as the trophectoderm and primitive endoderm are generated, which then form the basis of the placenta and the extraembryonic yolk sacs (Hogan et al. 1986). Once implanted, a subset of cells forms the expanding primitive ectoderm from which the embryo will develop (Hogan et al. 1986; Fleming et al. 1992). At this point of differentiation the embryo is at the 128/256 cell stage with most of its cells forming extraembryonic cell tissues (Hogan et al. 1986). Consequently, only a subset of all mitochondria present in the zygote will contribute to the mitochondrial pool in the embryo proper (Fleming et al. 1992).

The apportionment of mitochondria to this subset of cells constitutes a numerical bottleneck during which rare mtDNA haplotypes, such as the paternally transmitted mtDNA, are potentially lost if present at low frequencies in the zygote (Bergstrom and Pritchard 1998). In other words, if paternal leakage occurs, the proportion of paternal mtDNA in the overall pool of mtDNA present in the zygote, is then linked to the

probability with which the paternal mtDNA will contribute to the subset of cells giving rise to the actual embryo. Consequently, the quantification of mtDNA in gametes, i.e. the characterization of the dilution effect in the zygote, is important if we are to better understand mitochondrial inheritance.

To date, our knowledge about the probable ratio of paternal to maternal mtDNA in the zygote is limited to two mammalian species. Gametes of mice were estimated to contain 10 to 75 and $1.6x10^5$ mtDNA molecules per single sperm and oocyte, respectively (Hecht et al. 1984; Shitara et al. 2000; Steuerwald et al. 2000). Human sperm were estimated to contain 1.4 to 6.8 molecules (May-Panloup et al. 2003; Amaral et al. 2007) and oocytes 1.93 to $7.95x10^5$ mtDNAs (Steuerwald et al. 2000; Reynier et al. 2001; Barritt et al. 2002; Chan et al. 2005; May-Panloup et al. 2005a; Santos et al. 2006). These estimates lead to ratios of $1:5.68x10^5$ to $1: 2.84x10^4$ of paternal to maternal mtDNA in human and $1:1.6x10^4$ to $1:2.1x10^3$ in mouse zygotes if paternal mtDNA enters the egg upon fertilization.

Considering differences in terms of life-history and reproductive strategies within the animal kingdom, with particular emphasis on gamete investment and gamete morphology, the ratio of paternal to maternal mtDNA is expected to vary. If this is indeed the case, taxa showing higher ratios of paternal to maternal mtDNA in zygotes might be more prone to paternal leakage than others. Therefore, investigating this dilution effect derived from mtDNA content of gametes in a systematic way (i.e. within different taxa) could provide valuable insights into the broader patterns of mtDNA inheritance.

Here, we determine the mtDNA content of salmon oocytes and build on earlier work examining the mtDNA content of sperm, which enables us to estimate the mtDNA ratio of paternal to maternal DNA in zygotes. The determination of this dilution effect is the first outside the mammalian system.

5.3 Material and Methods

5.3.1 Oocyte samples

Samples were collected during the spawning season in April and May 2005 in collaboration with the NIWA Silverstream Hatchery, located north of Christchurch on the Kaiapoi River. Eggs of 17 semi-wild returns (individuals are released into the wild and return for spawning to the hatchery) were collected by abdominal incision following standard husbandry procedures (McIntyre and Stickney 1991). Samples were stored in 70% EtOH at -20ºC until analysis.

5.3.2 Preparation of DNA

DNA was extracted from single eggs in 1.5ml extraction buffer, containing 4 M urea, 1% Tween 20, 1% Nonidet P-40, 5% Chelex-100 and Proteinase K (0.4µg/µl) (Aranishi 2006). To improve the digest, eggs were disrupted using a sterile glass rod. Samples were then incubated using a thermal shaker for six hours at 55ºC (shaking intervals of 5 sec at 1 400 rpm every 10 min). After incubation samples were boiled for 8 min, centrifuged for 2 min at 20 000g, diluted 1:40 in TE (10mM Tris, 1mM EDTA, pH 8) and stored at -20ºC. DNA extracts were not further purified to avoid loss of template during the purification process. To exclude the presence of inhibitory components in DNA extracts, the amplification efficiency of sample DNA was assessed through standard curves derived from amplifications of two-fold serial dilutions.

5.3.3 External standard

To quantify the number of mtDNA copies in single oocytes, we used a 314 bp fragment of the mitochondrial NADH dehydrogenase subunit 1 (*mt-nd1*) gene as external standard. PCR reactions were carried out with the forward primer ND1aF (5`-GGTAATTGCGAGAGGCCTAA-3`) and the reverse primer ND1R1 (5`-GTAAGGGCAAGTATGGGTGT-3`) under standard conditions with 25ng of whole genomic DNA in 25µl reactions (primers according to NCBI: NC_002980). PCR amplifications consisted of 2 min initial denaturation and 30 cycles of denaturation at 94ºC for 15 sec, annealing at 57ºC for 20 sec and elongation at 72ºC for 20 sec.

The PCR product was cloned using GeneJet^{TM} PCR cloning kit (Fermentas, Burlington, Canada) and the recombinant plasmid was purified using PureLink^{TM} Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, CA, USA). Clones were analyzed in-house on a capillary ABI3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA). The purity of the recombinant plasmid was checked by spectrophotometry using a ND-1000 NanoDrop (NanoDrop Tech., Wilmington, DE, USA) and the absence of residual genomic DNA by gel electrophoresis. The recombinant plasmid was 3442 bp in length (314 bp insert and 3128 bp vector pJet1), and 1µg of purified DNA was estimated to contain 2.70 x 10^{11} molecules (http://molbiol.edu.ru/eng/scripts/h01_07.html). Ten-fold serial dilutions (10^2 copies/µl to 10^7 copies/µl) were prepared, aliquoted and stored at -20ºC.

5.3.4 Quantification of the mtDNA content in oocytes

Samples were analyzed using a Mx3000P Q-PCR system (Stratagene, Garden Grove, CA, USA) in combination with the Fast Start SYBR Green Master mix (Roche, Basel, Switzerland). Sample and reference DNA were analyzed in 20µl reaction volumes, containing 1x Fast Start SYBR Green Master mix, 1µl DNA and 10pmol of each primer. Q-PCR amplifications consisted of initial denaturation for 10 min at 95ºC, followed by 36 cycles with 20 sec denaturation at 95ºC, annealing for 20 sec at 57ºC and elongation at 72ºC for 20 sec. The change in SYBR green fluorescence intensity was measured at the end of each extension step. After 36 cycles a melting curve analysis was performed to screen the reaction for non-specific amplification. To exclude the amplification of nuclear pseudogenes, we performed several BLAST searches using the mtDNA sequence we quantified in our study to search for similar sequences in salmonid databases. The BLAST searches of the mitochondrial *mt-nd1* sequence were performed against rainbow trout (*O. mykiss*) in particular (http://web.uvic.ca/cbr/grasp/) and against salmonid databases in general (www.salmongenome.no, www.ncbi.nlm.nih.gov/blast/). This search did not recover any similar sequences other than known mitochondrial *mt-nd1* sequences.

Amplifications of the reference DNA (10-fold serial dilutions of the recombinant plasmid, 10^2 to 10^7 copies) were performed to produce a standard curve for the quantification of mtDNA copy number in our DNA samples. All samples were measured in triplicate and results were analyzed using the software MxPro, supplied with the instrument. The mtDNA content was calculated with consideration of the applied dilution factor of DNA extracts (40x) and the initial amount of extraction buffer (1500x). Therefore, results from Q-PCR experiments were corrected by the factor 60 000 for the final estimate of mtDNAs per oocyte.

5.4 Results

5.4.1 Applicability of the Q-PCR assay

Amplifications of the external standard (Figure 5.1) and serial dilutions of sample DNA (data not shown) were performed with efficiencies of 99.6% and 99.3%, respectively. Slopes of derived standard curves were -3.332 for the external standard (Figure 5.2) and -3.333 for sample DNA (data not shown). As a measure of accuracy, the high similarities of slopes and efficiencies between standard and sample DNA confirmed the applicability of this Q-PCR to estimate the mtDNA content of oocytes.

5.4.2 Reproducibility of the Q-PCR assay

To assess the reproducibility of mtDNA quantifications, three oocytes with varying mtDNA contents were analyzed ten times. Amplifications of these samples resulted in highly repeatable estimates for the mtDNA content. Estimates for the number of mtDNAs per oocyte were between 2.29×10^9 and 3.24×10^9 for three different oocytes and the coefficient of variance (CV) was 4.86% for oocyte one, 1.86% and 1.18% for oocytes two and three, respectively (Table 5.1). Again, low CVs confirmed the utility of the Q-PCR assay and the highly reproducible nature of the experimental measures.

5.4.3 MtDNA content of oocytes

This Q-PCR assay was applied to measure the mtDNA content in oocytes from 17 different individuals. For each individual 5 oocytes were analyzed, each in triplicate. Table 5.2 shows the summarized results for each individual. The mtDNA content of oocytes per individual was calculated by the mean mtDNA content of 5 oocytes. The overall mean mtDNA content for these oocytes was $3.15x10^9 \pm 9.98x10^8$ molecules with an average coefficient of variance (CV) of 4.02% (Table 5.2).

The mitochondrial DNA content between different oocytes from the same individual varied moderately with the highest variation of 18.35% for individual 05-243 and the lowest variation of 2.51% for individual 05-268 (Table 5.2). The overall mean inter-oocyte variation within an individual was 8.03% (Table 5.2). The lowest detected mtDNA load was $9.41x10^8$ molecules per oocyte and the highest level was $7.65x10^9$ molecules per oocyte, leading to an overall inter-oocyte variation among individuals of 43.47% (Table 5.2).

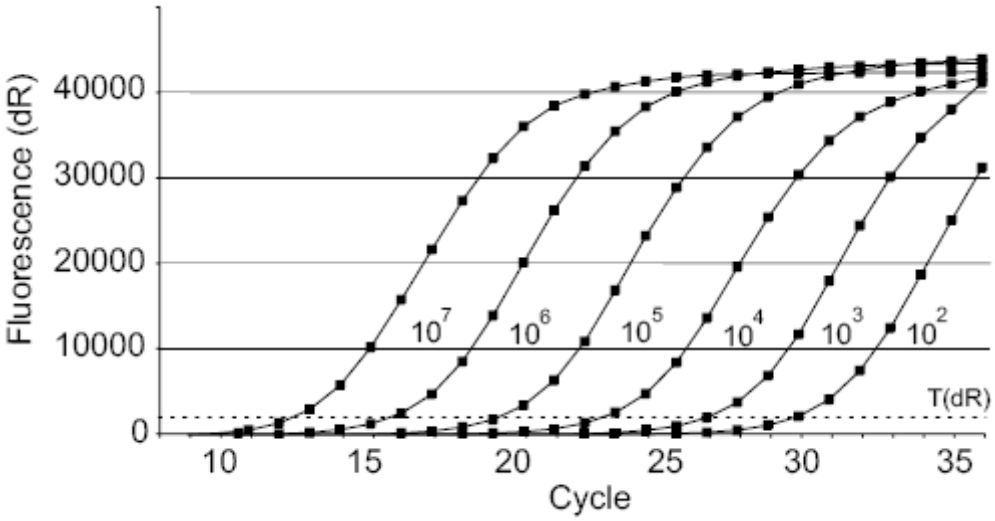

Figure 5.1: Amplification plots of the external standard. Amplifications plots of the ten-fold dilution series of the external standard for the estimation of mtDNAs per oocyte. Relative fluorescence (dR) plotted against cycle number. T(dR) = threshold fluorescence.

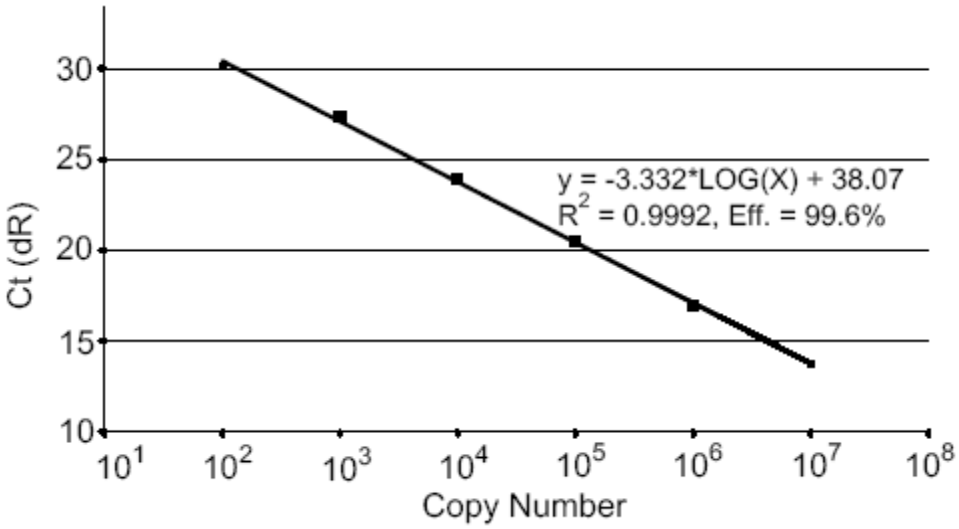

Figure 5.2: Standard curve of the external standard. Standard curve derived from amplification plots of ten-fold dilution series of the external standard. Threshold cycle plotted against initial amount of template (copy number). Ct(dR) = threshold cycle.

Table 5.1: Intra-assay variance of Q-PCR experiments. To reveal the measurement error of the applied Q-PCR experiments and to evaluate the reproducibility of the measurements, three different oocytes were measured ten times (mtDNA content $x10^9$).

Oocyte	1	2	3
	2.20	2.91	3.25
	2.40	2.90	3.22
	2.40	2.87	3.27
	2.22	2.97	3.30
	2.40	2.97	3.21
	2.41	3.00	3.22
	2.12	2.99	3.18
	2.26	2.87	3.29
	2.20	2.86	3.22
	2.25	2.97	3.22
Mean	2.29	2.93	3.24
Stdv[1]	0.11	0.05	0.04
CV(%)[2]	4.68	1.86	1.18

[1]Standard deviation [2]Coefficient of variance

Table 5.2: Mean mtDNA load of oocytes per individual. Mean mtDNA copy number of five oocytes per individual among 17 individuals of New Zeland chinook salmon. The average coefficient of variance (CV_{av}) documents the mean measurement error observed during the determination of mtDNA content for five oocytes per individual. The inter-oocyte coefficient of variance (CV_{i-o}) documents the variability of mtDNA content among five oocytes for each individual.

	Mean for 5 oocytes per individual			Inter-oocyte variation
Individual	MtDNA content [x10^9]	Stdv [x10^8]	CV_{av}(%)[1]	CV_{i-o}(%)[2]
05-201	2.82	0.91	3.23	7.72
05-208	1.83	0.55	3.01	10.71
05-209	3.39	0.92	2.72	5.51
05-213	2.32	1.08	4.63	4.49
05-214	1.40	7.67	5.50	13.39
05-227	3.78	1.34	3.54	5.32
05-239	3.87	1.68	4.34	9.60
05-243	1.12	0.41	3.69	18.35
05-254	3.84	1.59	4.15	3.51
05-256	4.07	1.78	4.37	13.96
05-263	2.90	0.99	3.40	4.49
05-268	2.86	1.25	4.36	2.51
05-275	3.16	1.53	4.85	14.55
05-280	3.96	1.34	3.39	6.59
05-283	2.19	0.78	3.59	3.36
05-285	3.10	1.17	3.77	4.44
05-357	6.98	4.00	5.73	7.95
Mean	3.15	1.30	4.02	8.03

[1]Average coefficient of variance for 5 oocytes; [2]Inter-oocyte coefficient of variance among 5 oocytes per individual

5.5 Discussion

This study found the average mtDNA content of oocytes to be $3.15x10^9 \pm 9.98x10^8$ among 17 individuals of New Zealand chinook salmon. With an average of $3.67x10^5$ mtDNAs per mammalian oocyte (Steuerwald et al. 2000; Reynier et al. 2001; Barritt et al. 2002; Chan et al. 2005; May-Panloup et al. 2005a; May-Panloup et al. 2005b; Santos et al. 2006) the estimated mtDNA content per salmon oocyte is approximately three to four orders of magnitude higher than that reported for mammals.

The number of mitochondria per salmon oocyte, or teleost oocytes in general, has not been determined but this number has been estimated by microscopy in two amphibians, which provides a point of reference for another taxa undergoing external development (Romek and Krzysztofowicz 2005). Stereological analysis of mitochondria in mature oocytes of a frog and a toad revealed an average of $1.12x10^8$ and $1.07x10^8$ mitochondria per oocyte, respectively (Romek and Krzysztofowicz 2005). According to these estimates the number of mitochondria per oocyte is approximately three times higher than that of mammalian oocytes (Piko and Matsumoto 1976; Shoubridge and Wai 2007). If we then consider that New Zealand chinook salmon oocytes are on average 4.5 to 5.5mm in diameter compared to 1.2 to 1.3mm for *Xenopus* oocytes (James 1972), the number of mitochondria per salmon oocyte might even lie well above estimates for amphibians.

The difference in mtDNA content between mammalian and salmon oocytes is likely to reflect the fundamental life history and developmental distinctions between these taxa. Mammalian embryos are exposed to an environment with high levels of nutrients, such as pyruvate, glucose, lactate and amino acids within the female reproductive tract (Gardner et al. 2002; Dumollard et al. 2007). These nutrients are directly utilized by the developing embryo to satisfy its metabolic requirements (Houghton and Leese 2004;

Dumollard et al. 2007). Oxidative metabolism, governed by mitochondria, is limited during early embryogenesis and is assumed to protect the developing embryo from mtDNA damage caused by free radicals (produced through the oxidative phosphorylation [OXPHOS] pathway) in mitochondria (Lenaz 1998; Leese 2002; Houghton and Leese 2004).

Teleosts instead develop externally, depending fully on nutrients present in the lipid and protein-rich yolk. The oxidation of yolk fatty acids serves here as the main source of energy (Ohkubo et al. 2006). Therefore, teleost mitochondria can be assumed to show higher activity during early embryogenesis as the oxidation process to metabolize fatty acids into pyruvate takes place in mitochondria (Stryer 1995). Considering that embryos of taxa showing external development depend to a higher degree on intra-oocyte mitochondrial activity compared to taxa showing internal development, the documented difference in mitochondrial load per oocyte is likely to be a necessity to meet the metabolic demands of externally developing embryos.

Taking into account that mitochondria of taxa showing external development are believed to have a higher metabolic activity, it is also possible that oocytes of such taxa not only have greater pools of mitochondria but probably also contain more mtDNAs per mitochondrion. With each mitochondrion containing multiple copies of the mitochondrial genome and considering that salmon oocytes might contain greater mitochondrial pools than amphibian oocytes (according to their size), mtDNA contents of salmon oocytes detected in this study are within the range of expectations.

However, the key focus of our work was to describe the dilution effect of paternal mtDNA in the fertilized zygote. Recently, we determined the mtDNA content of chinook salmon sperm to be 5.73 ± 2.28 per gamete and here, we estimated the average mtDNA content of salmon oocytes to be $3.15 \times 10^9 \pm 9.98 \times 10^8$. If these estimates are indicative and

if paternal mtDNA enters the egg upon fertilization without subsequent degradation, the ratio of paternal to maternal mtDNA is then $1:5.49x10^8$. Accordingly, the contribution of paternal mtDNA to the overall mtDNA pool in salmon zygotes is three to five orders of magnitude smaller compared to the mammalian system.

As for mammalian embryogenesis (Cao et al. 2007), the mtDNA content of teleost oocytes is believed to remain constant during early embryogenesis and mitochondria present in the oocyte are apportioned to the cells of the developing embryo with the majority assigned to extra-embryonic tissues (Fleming et al. 1992; Dumollard et al. 2007). Thus, a genetic bottleneck comparable to that described for mammalian embryogenesis is expected in teleosts. If species-specific mechanisms to prevent paternal leakage break down, the dilution of paternal mtDNA by maternal mtDNA might serve as efficient barrier to avoid the inheritance of paternally derived mtDNA to offspring. Consequently, paternal inheritance of mtDNA may be more likely per individual in mammals than in salmon.

Finally, our results indicate that the probability of paternal leakage occurring based on the dilution effect only, might be linked to taxa-specific life traits such as gamete investment, morphology, reproductive strategy (internal vs. external fertilization) and embryogenesis (internal vs. external development). Thus, as other taxa may be equally disparate from the mammalian system, it is desirable that further study be undertaken across a range of other taxa to better understand the mechanisms of mitochondrial inheritance.

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Chapter 6

6 Segregation of Mitochondrial DNA Occurs During Oogenesis, Rather than Embryogenesis in Chinook Salmon

6.1 Abstract

Mutations in the mitochondrial genome have been increasingly associated with disease. Generally, affected individuals are heteroplasmic for mtDNA and the expression of the disease phenotype is associated with the allele frequency of the mutated allele. Pedigree analyses in a wide range of species revealed that intergenerational shifts in mtDNA allele frequencies can be rapid and a return to homoplasmy can occur within a few generations. This observation has led to the idea of genetic bottlenecks acting on mtDNA copy number during early developmental stages. Whereas the size (number of segregating mtDNA molecules) of this bottleneck has been estimated for several species, information to elucidate at what developmental stage such a bottleneck may occur is both scarce and highly controversial. Both oogenesis and embryogenesis are characterized by cell proliferation and changes in mtDNA load and therefore believed to induce bottleneck effects. To estimate the size of this bottleneck in salmon and to determine whether such a bottleneck is likely to occur during oogenesis or embryogenesis, we examined the transmission of mtDNA variants to F1 offspring and oocytes within a pedigree of heteroplasmic chinook salmon. The mean number of segregating units between a mother's somatic tissue and oocytes was estimated to be 109.3 and from a mother's soma to F1 offspring's soma 105.4. Detected variances in allele frequency among oocytes were not significantly different from those in F1 offspring, strongly suggesting that segregation of mtDNA occurs during oogenesis with its completion before oocyte maturation. Accordingly, we found no evidence for significant mtDNA segregation and continuative change in allele frequency during embryogenesis.

6.2 Introduction

Animal mitochondrial DNA (mtDNA) is a circular double-stranded molecule, generally varying between 15 and 20 kb in length. Its gene content is highly conserved and typically encodes for 13 polypeptides involved in oxidative phosphorylation, 22 transfer RNAs and two ribosomal RNAs (Wolstenholme 1992). Unlike nuclear DNA, the inheritance of the mitochondrial genome is non-Mendelian and believed to follow a strict maternal mode of transmission (Hutchison et al. 1974; Giles et al. 1980; Birky 1995). As a consequence, an individual's mtDNA population is generally derived exclusively from the maternal parent and therefore is homoplasmic for one type of mtDNA. However, due to the release of free radicals during oxidative activity of mitochondria, mammalian mtDNA has generally an elevated mutation rate (compared to nuclear DNA) and is therefore highly polymorphic (Brown et al. 1979). As a result of such mutations, coupled with the large population size of mtDNAs in a cell (Robin and Wong 1988), new mtDNA haplotypes will arise and many individuals will subsequently harbor mtDNA populations with two, or probably more, distinct types of mtDNAs (heteroplasmy).

Despite an elevated mutation rate and the high population size of mtDNA in most cells, which leads to an expected accumulation of mutations (and therefore new haplotypes) over time, heteroplasmy seems to be less frequent in most species than expected. Pioneering work to unveil the mechanisms behind this contradictory observation was undertaken by Hauswirth and Laipis (1982) who showed in a pedigree of Holstein cows that intergenerational shifts in haplotype composition can be rapid and that in some cases, replacement of single haplotypes can occur within a few generations. This observation led to the assumptions that mtDNA is exposed to a genetic bottleneck, or at least a bottleneck effect, during early embryogenesis and/or oogenesis (Jansen and de Boer

1998; see also Figures 6.1 and 6.2). The presence of such a bottleneck is believed to act as a mechanism against the accumulation of deleterious mutations and 'mutational meltdown' (Lynch and Blanchard 1998; Shoubridge and Wai 2008) that would occur via Muller's ratchet in a molecule that is clonally inherited (Bergstrom and Pritchard 1998). This concept has recently received support with the demonstration that (i) this bottleneck can have a 'purifying' effect on the inheritance of mtDNA and therefore promote mitochondrial functionality in a strain of mutator mice (Stewart et al. 2008) and that (ii) severely mutated mtDNA may undergo selective elimination during oogenesis (Fan et al. 2008).

However, while there appears to be little contention about whether a bottleneck occurs or not, it remains unclear and the subject of much debate as to when, i.e. during oogenesis, embryogenesis or both, such a bottleneck might occur (Jenuth et al. 1996; Smith et al. 2002; Cao et al. 2007; Cree et al. 2008; Khrapko 2008; see also Figures 6.1 and 6.2). Both oogenesis and embryogenesis are characterized by extensive cell proliferation and small numbers of founder cells during which bottleneck effects might result in significant shifts in heteroplasmy levels (Hogan et al. 1986; Fleming et al. 1992; Poulton and Marchington 2002; Shoubridge and Wai 2007).

In embryogenesis, mitochondria are believed to be equally and randomly apportioned to the arising cells in the cleaving embryo (Figure 6.2). It has been demonstrated that the mtDNA content remains constant during early stages of embryogenesis in sea urchin, frogs, nematodes, fish and mice (Chase and Dawid 1972; Matsumoto et al. 1974; Piko and Matsumoto 1976; Piko and Taylor 1987; Ebert et al. 1988; Wang and Yan 1992; Larsson et al. 1998; Tsang and Lemire 2002; Thundathil et al. 2005; Cao et al. 2007; Cree et al. 2008). In mice, for example, replication of mtDNA does not commence before the early blastocyst stage (Piko and Taylor 1987). At this stage the

developing embryo has already undergone numerous cell divisions, with the mitochondria originally present in the zygote randomly apportioned to these cells. After first cell divisions, cell lineages such as the trophectoderm and primitive endoderm are formed which subsequently give rise to the placenta and extraembryonic yolk sacs (Hogan et al. 1986). After implantation of the embryo, a subset of cells (the arising inner cell mass, ICM) forms the expanding primitive ectoderm from which the embryo will develop (Hogan et al. 1986; Fleming et al. 1992). At this stage of development, the evolving embryo is at the 128/256 cell stage with most of its cells forming extraembryonic cell tissue (Hogan et al. 1986). Consequently, only a subset of mitochondria originally present in the zygote will contribute to the actual embryo proper, potentially introducing an intergenerational bottleneck effect.

A similar sampling effect which determines the mtDNA composition of the inner cell mass (ICM) and therefore that of the developing embryo, also determines the mtDNA founder population of the female germline. In mice, the development of the female germline begins with the specification of primordial germ cells (PGCs) in the primitive ectoderm (which derives from the ICM) at stage E6 to E7 during early embryogenesis (Hogan et al. 1986; Ginsburg et al. 1990; Shoubridge and Wai 2007). The number of founder cells or PGCs that give rise to the female germline is believed to be as low as 8 cells (Ginsburg et al. 1990). PGCs then gradually increase in number to approximately 200 at E9 to E9.5 (Ginsburg et al. 1990). These cells proliferate further and develop to oogonia before colonizing the developing gonads at E12-13, with each gonad containing 4,000 to 11,000 oogonia which give rise to approximately 25,000 primary oocytes (Tam and Snow 1981; Wartenberg et al. 1998). Note, that the number of germ cells during fetal development in humans is estimated to peak at ~7.0 $x10^6$, presenting an even greater expansion of the germ cell population (Jansen and de Boer 1998).

The increase in germ cell number occurs concomitantly with increasing mtDNA content per germ cell (Jansen and de Boer 1998; Cao et al. 2007; Shoubridge and Wai 2007; Cree et al. 2008; see also Figure 6.1). In both cases, human and mouse, the vast number of germ cells originates from a limited number of founder cells (PGCs), each of which contains approximately 10-100 mitochondria (Jansen and de Boer 1998; Cao et al. 2007; Shoubridge and Wai 2007; Figure 6.1). Recent findings also point to a moderate increase of mtDNA during maturation of PGCs (Cree et al. 2008). During subsequent cell proliferation, the number of mitochondria, and therefore the number of mtDNA molecules, increases to ~200 mitochondria in oogonia, 5,000 in primary oocytes and 100,000 in mature oocytes (Jansen and de Boer 1998; Shoubridge and Wai 2007; Figure 6.1). This vast expansion in cell number and mtDNA content during fetal life constitutes a typical bottleneck effect during which vegetative segregation and unequal partitioning of mitochondria potentially lead to sharp shifts in heteroplasmy/mutational levels among offspring.

To describe the mtDNA bottleneck effect, numerous studies have aimed to examine the intergenerational transmission of mutational loads or heteroplasmy levels between heteroplasmic mother/offspring pairs (Hauswirth and Laipis 1982; Solignac et al. 1984; Rand and Harrison 1986; Ashley et al. 1989; Koehler et al. 1991; Howell et al. 1992; Bendall et al. 1996; Jenuth et al. 1996; Cao et al. 2007; Cree et al. 2008; Stewart et al. 2008). By monitoring differences in haplotype composition between mother and offspring, the number of segregating units (N_e), i.e. the number of mtDNA molecules which is transmitted from mother to offspring, can be estimated. Such studies estimated N_e to be 20 to 163 in Holstein cows (discussed in Rand and Harrison 1986; Ashley et al. 1989), 87 to 395 in crickets (Rand and Harrison 1986), 370 to 740 in fruit flies (Solignac et al. 1984), 2

to 349 in humans (Howell et al. 1992; Bendall et al. 1996; Jenuth et al. 1996) and ~200 in mice (Jenuth et al. 1996).

Although the comparison of mother/offspring pairs allows for estimates of N_e between single generations, this approach fails to pinpoint at what stage such a bottleneck effect may occur. In fact, the only study to monitor changes in mutational load during different developmental stages including both oogenesis and embryogenesis to determine at which stage the bottleneck is likely to occur was undertaken a decade ago by Jenuth et al. (1996). Using a strain of heteroplasmic mice, Jenuth et al. (1996) compared the variance in heteroplasmy levels in both F1 offspring and mature oocytes to that observed in the parental generation. They observed no significant difference in heteroplasmy levels between F1 offspring and mature oocytes (Jenuth et al. 1996). Consequently, they excluded unequal partitioning and segregation of mtDNA during early embryogenesis as a major factor causing the differences in heteroplasmy levels between mother/offspring pairs and concluded that this process must occur at an earlier stage of development, i. e. during oogenesis (Jenuth et al. 1996). By observing similar variances of mutational load between mature oocytes and immature oocytes they further concluded that the bottleneck causing these shifts must occur at earlier stages of germline development, most likely during extensive cell proliferation from PGCs (~200) to oogonia (8,000 to 22,000) (Jenuth et al. 1996). Recent work, investigating the inheritance of severe mtDNA mutations in mice, confirmed these findings by documenting that shifts in allele frequencies observed in offspring were already observable in oocytes (Fan et al. 2008).

Contradicting these findings, Cree and co-workers (2008), also working on heteroplasmic mice, concluded that the bottleneck occurs mainly during embryogenesis. In their study on the inheritance of heteroplasmy, Cree and co-workers (2008) measured the mtDNA content of single cells at different stages of embryogenesis and oogenesis and used

a modified mathematical segregation model to predict the inheritance of heteroplasmy based on heteroplasmy levels in the parental generation (Cree et al. 2008). The accuracy of this model was then compared to actual measurements of intergenerational shifts in heteroplasmy levels in a strain of heteroplasmic mice combined with their measurements of mtDNA content in single cells. They found that 91% of the offspring had heteroplasmy levels that fell within the predicted 95% confidence interval of the model and that simulated amounts of mtDNA in their model are in accordance to actual measurements in the embryo (Cree et al. 2008). According to this model and the observed reduction of mtDNA, the variance in heteroplasmy levels observed among the offspring investigated is mainly caused by mtDNA segregation during pre- and early postimplantation development (~70%, during continuative cell proliferation of the ICM, see also Figure 6.2) and partly caused by mtDNA replication during oocyte maturation (30%, during continuative cell proliferation from PGCs to oocytes, see also Figure 6.1) (Cree et al. 2008).

These contradictory findings have reignited the debate surrounding the mtDNA bottleneck during early developmental stages and further studies are inevitable and needed if we are to understand better the mechanisms of the inheritance of heteroplasmy. Here, we follow the intergenerational transmission of mtDNA in heteroplasmic individuals of a non-mammalian vertebrate, New Zealand chinook salmon, to (i) examine the change in heteroplasmy levels from mother to oocytes, (ii) examine the change in heteroplasmy levels from mother to F1 offspring, (iii) determine the number of segregating units N_eOog between mother/oocyte pairs (i.e. N_e in oogenesis), and (iv) determine the number of segregating units N_eEmb between mother/offspring pairs (i.e. N_e in embryogenesis). These results provide an in depth insight into mechanisms of the transmission of heteroplasmy in chinook salmon, the first non-mammalian vertebrate to be examined in such comprehensive way, and enable us to determine the size of the genetic bottleneck, if

present, in both oogenesis and embryogenesis. Also, these results enable us to elucidate at what developmental stage the bottleneck is likely to occur, potentially adding important new data to the ongoing debate surrounding the timing and strength of mtDNA bottlenecks.

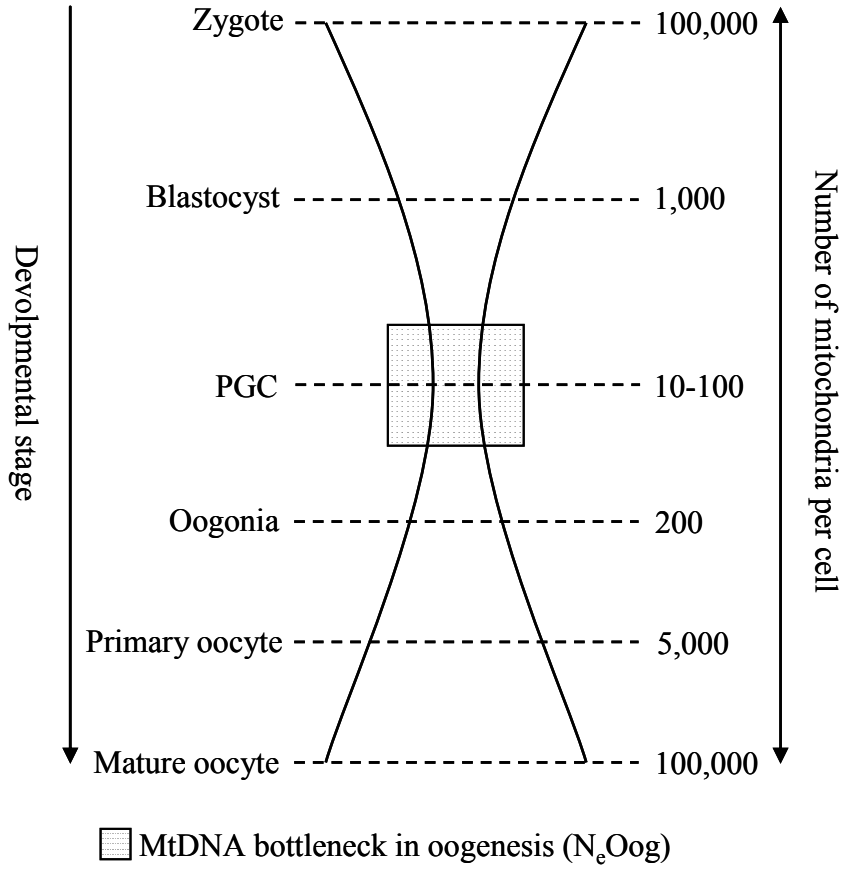

Figure 6.1: Schematic illustration of the mtDNA bottleneck during mammalian oogenesis with N_eOog depicting the bottleneck in oogenesis (number of segregating units, *i.e.* mtDNA molecules). Change in number of mitochondria per cell in embryogenesis and oogenesis. The number of mitochondria per cell decreases in the cleaving embryo to a minimum of 10-100 mitochondria per cell in early primordial germ cells (PGCs) which give rise to the female germ line. The sharp reduction of mitochondrial load from 100,000 mitochondria in zygotes to 10-100 mitochondria in primordial germ cells (PGCs) followed by a vast expansion during oocyte maturation potentially constitutes a genetic bottleneck (adapted and modified from Shoubridge and Wai 2007).

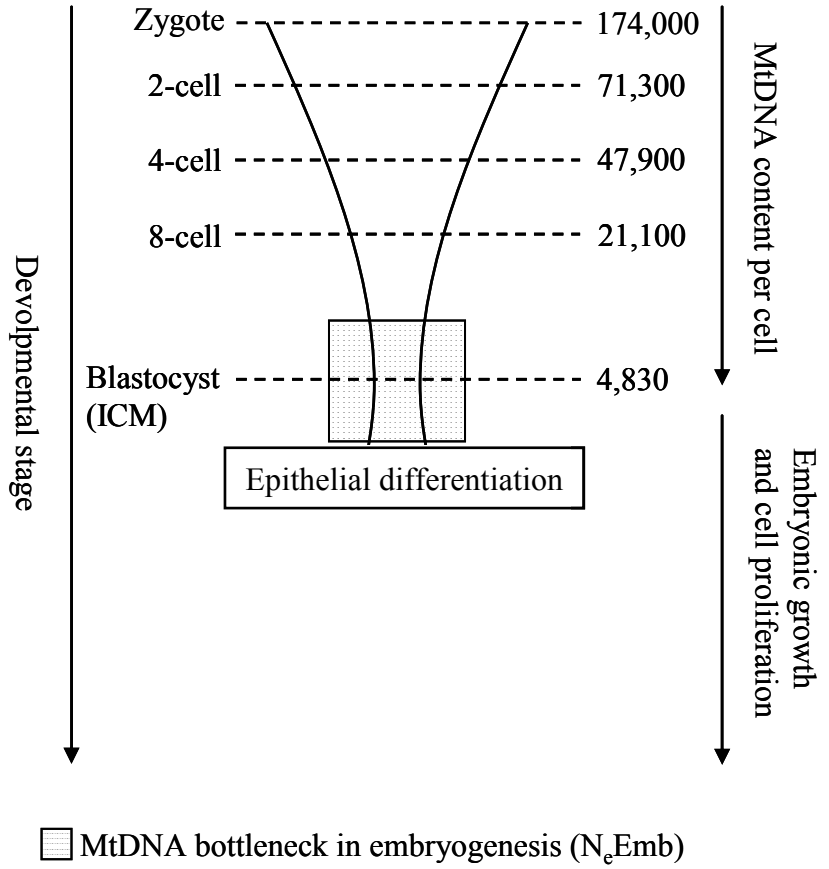

Figure 6.2: Schematic illustration of the mtDNA bottleneck during mammalian embryogenesis with N_eEmb defining the bottleneck (number of segregating mtDNA molecules). Change in number of mtDNA molecules per single cell during early stages of embryogenesis. The mtDNA content decreases from 174,000 in zygotes to 4,830 in cells of the blastocyst. Only a few cells of the blastocyst give rise to the inner cell mass (ICM) from which the embryo will subsequently develop. This sharp reduction in mtDNA content prior to epithelial differentiation potentially constitutes a genetic bottleneck (values for mtDNA content per cell adapted from Cao et al. 2007).

6.3 Material and Methods

6.3.1 Marker

Two heteroplasmic sites investigated in this work were detected previously within a hatchery population of New Zealand chinook salmon at the NIWA Silverstream hatchery (http://www.niwa.co.nz/rc/aqua/silverstream) in the mitochondrial gene *mt-nd1* at nucleotide positions 4149 and 4316 (NCBI:NC_002980; Metcalf et al. unpublished). The heteroplasmy at position 4149 is an A/G polymorphism (G = wildtype) and the heteroplasmy at position 4316 is a C/T polymorphism (T = wildtype). Both heteroplasmic sites are synonymous and were detected by Sanger sequencing and authenticated by cloning experiments to resolve both haplotypes.

6.3.2 Samples

Heteroplasmy levels among six female fish and both their ooctyes and F1 offspring were examined. Two females were heteroplasmic for nucleotide position 4316 and four individuals were heteroplasmic for position 4149. All husbandry work, necessary to conduct fertilization experiments and to rear offspring, was carried out during and after the spawning season in April and May 2005 at the Silverstream hatchery. To monitor the inheritance of heteroplasmy, F1 offspring were generated using the dry method between homoplasmic males and heteroplasmic females. Fertilized eggs were incubated and embryos reared until hatching in hatching jars following standard husbandry procedures

(Huet 1970; McIntyre and Stickney 1991). Both embryos and eggs were harvested and stored in 80% EtOH at -20ºC until analysis.

6.3.3 DNA extractions

F1 offspring: Whole genomic DNA was extracted from F1 offspring (1-2 mm tail tissue) in 350 µl extraction buffer, containing 5% Chelex-100, 100 mM NaCl, 50 mM Tris [pH 8.0], 1% SDS, 10 mM EDTA, 100µg/ml RNase A and 100µg/ml Proteinase K using a thermal shaker at 55ºC and 180 rpm for four hours. After incubation, cell debris was precipitated by centrifugation (4000 rpm, 3 min) and the supernatant was transferred into new reaction tubes containing equal amounts TE-Chelex-100 solution (5% Chelex-100, 10 mM Tris [pH 8.0], 1 mM EDTA). Dilutions were prepared (1:10) and stored at -20ºC until analysis.

Oocytes: DNA was extracted from single eggs in 1.5ml extraction buffer, containing 4 M urea, 1% Tween 20, 1% Nonidet P-40, 5% Chelex-100 and Proteinase K (0.4µg/µl) (Aranishi 2006). To improve the digest, eggs were disrupted using a sterile glass rod. Samples were then incubated using a thermal shaker for six hours at 55ºC (shaking intervals of 5 sec at 1 400 rpm every 10 min). After incubation samples were boiled for 8 min, centrifuged for 2 min at 20 000g, diluted 1:40 in TE (10mM Tris, 1mM EDTA, pH 8) and stored at -20ºC.

6.3.4 Pyrosequencing

Allele frequencies were determined using pyrosequencing. This work was undertaken in collaboration with Dr. Helen White at the National Genetics Reference Laboratory (Wessex, UK) at the Salisbury District Hospital.

PCR amplification and clean-up: The sequences of PCR and sequencing primers (Biomers, Ulm, Germany) used for each assay are listed in Table 6.1. Amplicons were generated in a 50µl reaction volume with 15pmol of forward and reverse PCR primers, 0.2mM dNTPs (Promega, Madison, USA), 1.5mM $MgCl_2$, 1X Buffer II (Applied Biosystems), 1U AmpliTaq Gold (Applied Biosystems, Foster City, USA) using approximately 10ng genomic DNA. PCR conditions for all reactions were 94°C for 7 min; 40 cycles with denaturation at 94°C for 30s, annealing at 57°C for 30s and elongation at 72°C for 30s; 1 cycle at 72°C for 7 min; and a final hold at 15°C.

Single-stranded biotinylated PCR products were prepared for sequencing using the Pyrosequencing™ Vacuum Prep Tool. Three microliter of Streptavidin Sepharose™ HP (Amersham, Little Chalfont, UK) was added to 37µl Binding buffer (10 mM Tris-HCl pH 7.6, 2M NaCl, 1 mM EDTA, 0.1% Tween 20) and mixed with 20µl PCR product and 20µl high purity water for 10 min at room temperature using a thermal shaker. The beads containing the immobilized templates were captured onto the filter probes after applying the vacuum and then washed with 70% ethanol for 5 sec, denaturation solution (0.2M NaOH) for 5 sec and washing buffer (10 mM Tris-Acetate pH 7.6) for 5 sec. The vacuum was then released and the beads released into a PSQ 96 Plate Low containing 45µl annealing buffer (20 mM Tris-Acetate, 2 mM $MgAc_2$ pH 7.6), 0.3µM sequencing primer (see Table 6.1). The samples were heated to 80°C for 2 min and then allowed to cool to room temperature.

Pyrosequencing reactions and data analysis: Pyrosequencing reactions were performed according to the manufacturer's instructions using the PSQ 96 SNP Reagent Kit containing the enzyme and substrate mixture and nucleotides (Pyrosequencing AB, Uppsala, Sweden). Assays were performed using the nucleotide dispensation orders shown in Table 6.1. The sample genotype and heteroplasmic state (%) were determined using the Allele Frequency Quantification (AQ) function in the SNP Software (Pyrosequencing AB, Uppsala, Sweden).

6.3.5 Mathematical analysis: Calculating the number N_e of segregating units

Mathematical modeling was carried out by Michael Woodhams using a model adapted from Hendy, Woodhams and co-workers (Hendy et al., under review). The applied model is a single sampling model. Due to the dynamics of mtDNA replication during embryogenesis and particularly oogenesis, a repeated sampling model was not considered. Due to limited or moderate mtDNA replication and cell proliferation during early germ line development (proliferation of PGCs) compared to vast cell expansion and mtDNA replication during development of oogonia and primary oocytes, the applicability of a repeated sampling model in whch sampling occurs during each cell division appears unlikely. There is no evidence for the validity of a single sampling model over a repeat sampling model, but based on the dynamics of mtDNA replication during oogenesis and embryogenesis, a single sampling model is favorable.

The model: Let the genome number (N) of a cell be the total number of mitochondrial genomes it contains, and the genome number of the bottleneck cell be N_b. Initially we consider the simplest model, where it is assumeed that once mitochondrial genesis restarts, all subsequent cells have genome number very much greater than N_b. We consider heteroplasmic cells, containing a mixture of genomes of types A and B. The *heteroplasmy number* (n) is defined as the number of type A genomes a cell contains, and the *heteroplasmy ratio p* to be the proportion of genomes that are of type A, so

$$p = n / N \tag{1}$$

If the initial egg has heteroplasmy ratio p, then the bottleneck cell has N_b genomes selected at random from this population, so its heteroplasmy number n_b will follow a binomial distribution characterized by N_b and p, having expected value

$$E(n_b) = N_b p \tag{2}$$

and variance

$$Var(n_b) = N_b p(1 - p). \tag{3}$$

Then the bottleneck heteroplasmy ratio p_b has

$$E(p_b) = p \tag{4}$$

and

$$Var(p_b) = p(1 - p) / N_b. \tag{5}$$

By the model assumption that all germ line cells after the bottleneck have large N (and hence small variance in p), the subsequent germ line cells, up to and including the egg in the next generation, have the same heteroplasmy ratio as the bottleneck cell. Under this model, the variation in heteroplasmy ratio between generations depends only on Nb. Reality is more complex: there may be several generations of cells with low genome

number; replacement of aging mitochondria can vary the heteroplasmy ratio over the lifetime of a cell, not just at cell division; the use of the binomial distribution implicitly assumes the types of the genomes in the daughter cell are independent of each other, but this is not true unless the genome number of the daughter cell is much less than the genome number of the mother cell. Despite all of this, we can measure the variance in heteroplasmy ratio p over one generation, and derive an effective bottleneck genome number from $N_{eff} = p(1\text{-}p)/Var(p)$. As these complications generally increase the generational heteroplasmy ratio variance compared to the simple model, we expect that N_{eff} will be somewhat less than the true bottleneck genome number.

In illustration, if we consider starting an egg with $p = 0.5$ and passing through two bottlenecks of $N = 1000$, then, after the first bottleneck, there is the heteroplasmy ratio p' with expected value $E(p') = p = 0.5$ and variance 0.5 x 0.5 / 1000 = 1 / 4000. After the second bottleneck, there is $E(p'') = p'$ and variance about the p' value of $p'(1 - p') / 1000 \approx 1 / 4000$, and therefore variance about the initial p' value of 1 / 2000. By comparison, a single bottleneck of $N = 500$ would have variance 0.5 x 0.5 / 5000 = 1 / 2000, i.e. two bottlenecks of $N = 1000$ are equivalent to a single bottleneck of $N_{eff} = 500$. The general rule for 'adding' bottlenecks is $1 / N_{eff} = 1 / N_1 + 1 / N_2 + \cdots$. This formula is exact for the diffusion limit (large N) described by Kimura (Kimura 1970) but looses accuracy for small N. The *drift distance d* is then defined by

$$d = 1/N. \qquad (6)$$

The drift distance is a measure of how much the heteroplasmy ratio can be expected to change. By the result above, drift distances are additive: passing through two $d = 0{:}001$ bottlenecks has the same effect as a single $d = 0{:}002$ bottleneck. Because of this property,

it is more convenient to deal with drift distances (d) rather than bottleneck genome numbers (N).

Simple analysis; theory: From the data in Table 6.2, the variance between mother and offspring heteroplasmy ratios can be estimated. If p_i is the heteroplasmy ratio of the i^{th}mother, and p_{ij} that of that mother's j^{th} (of M_i) offspring, then the estimator for the raw variance from the i^{th} mother is

$$\hat{\sigma}^2_{rawi} = \frac{1}{M_i}\sum_{j=1}^{M_i}(p_i - p_{ij})^2 \qquad (7)$$

The raw variance is a combination of the actual drift distance between mother and offspring, and the uncertainties in measurements of the heteroplasmy ratios:

$$\sigma^2_{raw} = \sigma^2_{mother} + \sigma^2_{drift} + \sigma^2_{offspring} \qquad (8)$$

According to this model, $\sigma^2_{drift} = dp(1-p)$, so in general, it cannot be assumed that the raw variances σ^2_{raw} are the same for each mother. However, in the salmon data, $p(1 - p)$ varies non-significantly from mother to mother (0.186 to 0.230), permitting to construct a single raw variance estimator for the entire data set:

$$\hat{\sigma}^2_{raw} = \frac{1}{\sum_i M_i}\sum_{i,j}(p_i - p_{ij})^2 \qquad (9)$$

To estimate σ^2_{drift} from this, we also need estimators $\hat{\sigma}^2_{mother}$ and $\hat{\sigma}^2_{offspring}$, then

$$\hat{\sigma}^2_{drift} = \hat{\sigma}^2_{raw} - \hat{\sigma}^2_{mother} - \hat{\sigma}^2_{offspring} \quad (10)$$

$$\hat{d} = \frac{\hat{\sigma}^2_{drift}}{p(1-p)} \quad (11)$$

As $\hat{\sigma}^2_{drift} = p(1-p)/N$ (by the binomial distribution) and $d = 1 / N$. To find confidence limits in d, the uncertainties in the estimators $\hat{\sigma}^2_{raw}$, $\hat{\sigma}^2_{mother}$ and $\hat{\sigma}^2_{offspring}$ need to be known. For $\hat{\sigma}^2_{raw}$, the standard result for the sample variance distribution can be applied,

$$\mathrm{var}(\hat{\sigma}^2) = \frac{(N-1)^2}{N^3}\mu_4 - \frac{(N-1)(N-3)}{N^3}\mu_2^2 \quad (12)$$

(http://mathworld.wolfram.com/SampleVarianceDistribution.html), (where μ_4 and μ_2 are moments of the distribution) which in the case of a Gaussian distribution becomes:

$$\mathrm{var}(\hat{\sigma}^2) = \frac{2(N-1)}{N2}\sigma^4, \quad (13)$$

where σ^2 is the (unknown) true variance between heteroplasmy measurements from the mother and heteroplasmy measurements from the offspring. We therefore estimate the variance of the variance as

$$\mathrm{var}(\hat{\sigma}^2_{raw}) = \frac{2(N-1)}{N2}\hat{\sigma}^4_{raw} \quad (14)$$

The situation for estimating $\hat{\sigma}^2_{mother}$ and $\hat{\sigma}^2_{offspring}$ is more complex: we have multiple sets of data (measurements from a single individual) where we assume that all data sets

have the same variance, but have different means. For example, heteroplasmy measurements were made on two fish, obtaining 0.38, 0.40, 0.42 (three measurements) from fish one and 0.79, 0.80, 0.81, 0.82, 0.83 from fish two. Assuming both fish were drawn from a Gaussian distribution with the same unknown variance σ^2, but with different (also unknown) means then the sample variance of fish one measurements is $s_1^2 = 0.0004$ and the variance in this (assuming Gaussian distribution) is

$$\mathrm{var}(s_1^2) = \frac{2(N-1)}{N2}\sigma^4 = \frac{4}{9}\sigma^4 .$$

For fish two we have $s_2^2 = 0.00025$ and

$$\mathrm{var}(s_2^2) = \frac{2(N-1)}{N2}\sigma^4 = \frac{8}{25}\sigma^4 .$$

Obtaining the best estimate of σ^2 in this way a weighted average of these two must be determined. Weighting is achieved by the inverse of the variance of each estimate:

$$s^2 = \left(\frac{9}{4}s_1^2 + \frac{25}{8}s_2^2\right) \Big/ \left(\frac{9}{4} + \frac{25}{8}\right) = 0.0003128$$

The variance of a linear combination of independent random variables *X* and *Y* is given by $var(aX + bY) = a^2var(X) + b^2var(Y)$. Applying this to s^2 (which is a linear combination of s_1^2 and s_2^2) we get

$$\text{var}(s^2) = \left[\frac{9}{4}/\left(\frac{9}{4}+\frac{25}{8}\right)\right]^2 \text{var}(s_1^2) + \left[\frac{25}{8}\left(\frac{9}{4}+\frac{25}{8}\right)\right]^2 \text{var}(s_2^2)$$

$$= \left[\frac{9}{4}/\left(\frac{9}{4}+\frac{25}{8}\right)\right]^2 \frac{4}{9}\sigma^4 + \left[\frac{25}{8}\left(\frac{9}{4}+\frac{25}{8}\right)\right]^2 \frac{8}{25}\sigma^4$$

$$= \sigma^4 / \left(\frac{9}{4}+\frac{25}{8}\right)$$

$$= = \frac{8}{43}\sigma^4$$

Substituting s^2 for σ^2, we estimate the variance of s^2 as $\text{var}(s^2) \approx \frac{8}{43} 0.000313^2 = 1.820x10^{-8}$.

Application: The method described above is able to accommodate the situation where the measurement uncertainty differs between samples from the mother and samples from the offspring. Without considerable elaboration, it cannot accommodate different samples from within the same group having different uncertainties. Some of the offspring have been measured only once, and some three times. If we were to use the mean values for the offspring, which have been measured three times, these measurements would be more accurate than the rest. To keep things simple, where an offspring has multiple measurements, only the first is used for finding the mother-offspring difference and hence the raw variance $\hat{\sigma}^2_{raw}$. The additional measurements are used only for estimating $\hat{\sigma}^2_{offspring}$.

The measurement variance for eggs is $\hat{\sigma}^2_{eggs} = 6.2199x10^{-4}$, $\text{var}(\hat{\sigma}^2_{eggs}) = 1.6640x10^{-8}$. This corresponds to a measurement uncertainty of $\sigma^2_{eggs} = 0.0249 \pm 0.0026 \quad \left(= \sqrt{\hat{\sigma}^2_{eggs}} \pm 0.5\sqrt{\text{var}(\hat{\sigma}^2_{eggs}) / \sigma^2_{eggs}}\right)$. Similarly, the measurement

uncertainty for the maternal individuals is $\hat{\sigma}_{mother} = 0.0114 \pm 0.0016$ and for offspring $\hat{\sigma}_{offspring} = 0.0128 \pm 0.0011$. This shows that measurement uncertainties are larger for eggs than for mothers or offspring, but there is no evidence for a difference in the measurement uncertainties between mothers and offspring. It was therefore assumed that the measurement uncertainties for mothers and offspring are the same, and the data was combined for a single estimate: $\hat{\sigma}_{offspring,mothers} = 1.5382x10^{-4}$, $\text{var}(\hat{\sigma}^2_{offspring,mothers}) = 5.26x10^{-10}$.

Comparing maternal samples to eggs, for each mother-egg pair the difference between the mean of three heteroplasmy measurements of the mother and the first heteroplasmy measurement of the egg was determined. The mean square of these differences then gives us $\sigma^2_{raw,eggs} = 2.7023 \ x10^{-3}$ and $\text{var}(\sigma^2_{raw,eggs}) = 3.9427 \ x10^{-7}$ from 37 egg measurements, corresponding to a root-mean-square (RMS) difference of 0.0520 ± 0.0060. For offspring, the numbers are $\sigma^2_{raw,offsring} = 2.2599x10^{-3}$ and $\text{var}(\sigma^2_{raw,offspring}) = 1.7024x10^{-7}$ from 60 offspring measurements (RMS difference 0.0475 ± 0:0043).

To find the drift variance for the eggs, we need to subtract the measurement variance from the raw variance. As the mother's heteroplasmy measurement is the mean of three values (each with uncertainty $\hat{\sigma}_{offspring,mothers}$), the variance is

$$\hat{\sigma}^2_{drift,eggs} = \hat{\sigma}^2_{raw,eggs} - \hat{\sigma}^2_{offspring,mother}/3 - \hat{\sigma}^2_{eggs} \pm \sqrt{\text{var}(\sigma^2_{raw,eggs}) + \text{var}(\sigma^2_{offspring,mother})/9 + \text{var}(\hat{\sigma}^2_{eggs})}$$

$$= 2.2599x10^{-3} - 1.5382x10^{-4}/3 - 6.2199x10^{-4} \pm \sqrt{3.9472x10^{-7} + 5.26x10^{-10}/9 + 1}$$

$$= 2.03x10^{-3} \pm 0.35x10^{-3}$$

Offspring heteroplasmies were each taken from a single measurement with uncertainty $\hat{\sigma}_{offspring,mothers}$, so

$$\hat{\sigma}^2_{drift,offspring} = \hat{\sigma}^2_{raw,offspring} - \hat{\sigma}^2_{offspring,mother}/3 - \hat{\sigma}^2_{offspring} \pm \sqrt{\mathrm{var}(\sigma^2_{raw,eggs}) + \mathrm{var}(\sigma^2_{offspring,mother})/9 + \mathrm{var}(\hat{\sigma}^2_{offspring})}$$

$$= 2.2599x10^{-3} - 1.5382x10^{-4}x4/3 \pm \sqrt{1.7024x10^{-7} + 5.26x10^{-10}x10/9}$$

$$= 2.05x10^{-3} \pm 0.41x10^{-3}$$

$\hat{\sigma}^2_{drift,eggs}$ and $\hat{\sigma}^2_{drift,offspring}$ are in good agreement. Therefore there is no evidence for any additional random drift in heteroplasmy ratios between the egg stage and the offspring stage, *i.e* during embryogenesis. Equivalently stated, there is no bottleneck in genomes-per-cell between the egg and the offspring. On the assumption that $\hat{\sigma}^2_{drift,eggs}$ and $\hat{\sigma}^2_{drift,offspring}$ measure the same value, we can form a weighted mean of these two values:

$$\hat{\sigma}^2_{drift} = (2.05 \pm 0.35)x10^{-3}$$

This can be converted into an estimate of *d* by equation 11. The mean value of $p(1-p)$ for the six mothers is 0.214, so

$$\hat{d} = \frac{\hat{\sigma}^2_{drift}}{p(1-p)} = \frac{(2.05 \pm 0.35)x10^{-3}}{0.214} = (9.56 \pm 1.62)x10^{-3} \qquad (15)$$

which in turn implies $N = 105 \pm 18$. It seems a reasonable assumption that the posterior probability distribution for $\hat{d}$ is approximately Gaussian, then $N = 1 / d$ will have a skewed distribution. Once this is accounted for, the 95% confidence limits on N are 70.3 to 153.1.

Limitations of the Simple Method: The analysis described above has a great advantage of being computationally easy, but has a number of limitations and drawbacks. In many places Gaussian distributions are assumed, so that knowledge of the mean and variance fully describes the distribution. In reality, some of these distributions are skewed, *e.g.* a Bayesian likelihood analysis with flat prior gives a posterior distribution on $\hat{\sigma}^2_{mother,offspring}$ with skewed towards a heavy tail for large $\hat{\sigma}^2_{mother,offspring}$ values.

The calculation of $\text{var}(\hat{\sigma}^2_{raw})$ assumes all measurements of mother-offspring heteroplasmy difference are independent - but the mother heteroplasmy measurements are reused multiple times. When there is a triplet measurement of an egg or offspring, the calculation of $\hat{\sigma}^2_{raw}$ uses only the first measurement, resulting in a reduction in precision. Using the mean of the three measurements and then giving these measurements greater weight in the calculation of $\hat{\sigma}^2_{raw}$, can accounted for this reduction in precision.

As noted above, an overall average of $p(1-p)$ from the mothers is used. Greater accuracy can be achieved by calculating $\hat{\sigma}^2_{drift}$ and $\text{var}(\hat{\sigma}^2_{drift})$ independently for each mother, and then combining them into a single weighted average.

Table 6.1: Primer and target sequences and dispensation order for pyrosequencing to determine allele frequencies for two heteroplasmic sites.

Heteroplasmy	4149[1]	4316[1]
Forward PCR primer[2]	CTTCCCCCTTTCTATTCCTCG	CCGAGCAGTAGCACAAACCATTT
Reverse PCR primer	GCTCGGAGGGCTCCAATTA	GGCCACGTTGAAGGTTTGAA
Sequencing primer	TGCAAGTACAAATAGTACCC	GAGTAAACCCCCCCG
Sequence to analyze	CC/TAGGTTA AGATCTGTAAC	TGA/GTGATA ATCACGCTAA GTA
Dispensation order	GCTCAGTAG	CTGACTGAT

[1]nucleotide position; [2] biotinylated

6.4 Results

Heteroplasmy levels of female parents were measured in triplicate and ranged between 24.7% and 67.3% for the mutant allele (Table 6.2). Standard deviations of these measurements varied between 0.4% and 1.8% (Table 6.2). To monitor the transmission of the parental heteroplasmy during oogenesis and embryogenesis, DNA from 60 ooytes (ten per parental female) and 60 F1 offspring (ten per parental female) was extracted.

The allele frequency of the mutant allele was measured successfully for 41 of 60 oocytes and all 60 offspring (Table 6.2). Detected allele frequencies among oocytes and F1 offspring deviated moderately from that of parental females with heteroplasmy shifts of up to 22.7% and 10.6% for oocytes and offspring, respectively. Within cohorts of samples (i.e. oocytes or F1 offspring) of single females, differences in allele frequency between single samples were as high as 40.9% for oocytes and 14.9% for F1 offspring. The greatest shift among samples investigated was detected for oocyte 6 (female 163) for which a return to homoplasmy (for the mutant allele) was observed (Table 6.2). Statistical analysis of allele frequencies between oocytes and F1 offspring per female parent showed that variations in allele frequency were not significantly different between mature oocytes and F1 offspring (Table 6.3).

Mathematical analysis of the binominal distribution of mutant allele frequencies to infer the size of potential bottlenecks (i.e. N_eOog and N_eEmb) followed frequency measurements and was based on detected variances and standard deviations among offspring and F1 oocytes, and standard deviations in measurement error. To determine the measurement error, eleven oocyte samples and 15 F1 offspring samples were chosen in a random fashion and mutant allele frequencies of these samples were measured in triplicate (Table 6.4). The average value of detected standard deviations within oocytes or F1 offspring was then considered as average measurement error for either type of sample

(Table 6.4). The mean measurement error was found to be 2.12% and 1.11% for oocytes and F1 offspring, respectively (Table 6.4). The difference in measurement error between oocytes and F1 offspring was not significant (Mann-Whitney test; $U = 129.0$; $z = 2.41$; $P_1 = 0.0158$; $P_2 = 0.0079$)

The number N_eOog of segregating units from mother to mature oocytes was estimated to be 109.3 (median = 109.3; $62.4 < N_eOog < 189.6$; 95% confidence interval) and the number N_eEmb of segregating units between mother and F1 offspring was found to be 105.4 (median = 105.4; $70.3 < N_eEmb < 153.1$; 95% confidence interval).

Table 6.2: Allele frequencies of the mutant allele of female parents, their oocytes and F1 offspring (all values in [%]). Six females with ten oocytes and F1 offspring per female were examined. Females 214, 256, 263, 275 were heteroplasmic at nucleotide position 4149 and females 272 and 357 were heteroplasmic at nucleotide position 4316 (NCBI:NC_002980). To reveal the measurement error a subset of samples was chosen in a random fashion and analyzed in triplicate.

Heteroplasmic site	4149				4316	
Female parent	05-214	05-256	05-263	05-275	05-272	05-357
	64 ± 1.0	32.5 ± 0.6	67.3 ± 0.4	32.1 ± 1.8	24.7 ± 1.1	30.4 ± 1.2
Oocyte 1	-	-	59.1	29.7 ± 1.5	32.3	30.0
Oocyte 2	-	37.4	69.6	-	26.4 ± 1.7	26.3
Oocyte 3	59.7	-	64.8	33.2	22.2	34.3
Oocyte 4	63.0	-	-	25.2 ± 2.9	22.8	-
Oocyte 5	65.4	33.4	63.0	-	19.9	33.3 ± 1.7
Oocyte 6	59.7	-	100.0	-	-	-
Oocyte 7	59.5 ± 2.3	-	-	-	25.7 ± 1.7	28.4
Oocyte 8	69.0	35 ± 2.4	70.0	24.0	15.5	27.4 ± 4.5
Oocyte 9	50.0	32.9	-	24.3 ± 1.8	18.2	-
Oocyte 10	59.3 ± 0.6	-	64.5	29.8	17.2 ± 2.5	23.6
Mean	60.7	34.7	70.1	27.7	22.2	29.0
Stdv*	5.6	2.0	13.7	3.7	5.3	3.8
Variance	30.9	4.1	187.5	14.0	27.9	14.5
F1 offspring 1	64.9	40.0 ± 0.4	76.5	33.9	27.6	26.8 ± 1.8
F1 offspring 2	73.6	28.7	69.8 ± 0.2	29.4	27.3	40.8
F1 offspring 3	74.6 ± 0.7	33.2	64.8	28.8	22.7 ± 0.7	27.8
F1 offspring 4	74.9	31.8 ± 2.4	69.5	27.0	19	27.8 ± 1.0
F1 offspring 5	64.0	33.4	66.9	30.9 ± 2.0	15.2	31
F1 offspring 6	62.6 ± 1.1	33.3	72.9	34.7	18.6 ± 0.4	33.4
F1 offspring 7	61.8	33.8	64.9 ± 1.1	35.9	24.3	27.5
F1 offspring 8	59.7	31.1	69.4	33.4 ± 2.1	17.3	36.9
F1 offspring 9	64.0	33.3 ± 0.9	64.2	27.8	25.4	31.0 ± 1.8
F1 offspring 10	60.6	41.3	69.7 ± 1.4	38.9	15.4	26.7
Mean	66.1	34.0	68.9	32.2	21.3	31.0
Stdv*	5.7	3.9	4.4	4.4	5.3	5.3
Variance	32.9	15.0	19.5	19.0	27.8	28.4

* Standard deviation; - = no data available

Table 6.3: Mann-Whitney test between allele frequencies detected in oocyte and F1 offspring samples. Results of Mann-Whitney test to examine if allele frequencies of the mutant allele documented for oocytes were significantly different from those revealed for F1 offspring among six females investigated.

Female	U	z	P_1	P_2
05-214	61	1.8659	0.0676	0.0338
05-256	25.5	0.7778	0.4535	0.2268
05-263	43.5	0.8295	0.4173	0.2087
05-272	45	1.6270	0.1179	0.0589
05-275	49	0.3266	0.7802	0.3901
05-357	43	0.7807	0.4747	0.2374

P_1 (one-tailed), P_2(two-tailed)

Table 6.4: Determination of measurement error. Measurement error derived from triplicate measurements of mutational load among randomly chosen F1 offspring and oocyte samples.

Sample	AF_{mut}[%]			ME [%]	Sample	AF_{mut}[%]			ME [%]
	R1	R2	R3			R1	R2	R3	
05-214 oocyte7	57.8	61.1	60.1	1.69	05-214 F1 offspring 3	75.2	74.7	73.8	0.71
05-214 oocyte10	60.8	59.7	58.9	0.95	05-214 F1 offspring 6	61.4	63.5	62.8	1.07
05-256 oocyte8	35.7	37	32.3	2.43	05-256 F1 offspring 1	39.7	40.4	39.9	0.36
05-275 oocyte1	30.9	28	30.2	1.51	05-256 F1 offspring 4	34.4	29.7	31.3	2.39
05-275 oocyte4	26.5	27.2	21.8	2.94	05-256 F1 offspring 9	32.7	34.4	32.9	0.93
05-275 oocyte9	24.2	22.5	26.1	1.80	05-263 F1 offspring 2	69.7	69.8	70	0.15
05-272 oocyte2	28.3	25.5	25.3	1.68	05-263 F1 offspring 7	63.7	65.8	65.2	1.08
05-272 oocyte7	27.3	24	25.8	1.65	05-263 F1 offspring 10	69.7	69.8	70	0.15
05-272 oocyte10	19.7	17.1	14.7	2.50	05-275 F1 offspring 5	29.4	30.1	33.2	2.02
05-357 oocyte5	34	34.5	31.4	1.66	05-275 F1 offspring 8	35.7	31.5	33	2.13
05-357 oocyte8	32.5	23.9	25.9	4.50	05-272 F1 offspring 3	23.4	22	22.6	0.70
Mean				2.12	05-272 F1 offspring 6	18.9	18.8	18.1	0.44
					05-357 F1 offspring 1	27.8	27.9	24.8	1.76
					05-357 F1 offspring 4	26.9	27.8	28.8	0.95
					05-357 F1 offspring 9	33	30.3	29.6	1.80
					Mean				1.11

AFmut: allele frequency of mutant allele; R1-R3: replicate 1-3; ME: measurement error

6.5 Discussion

This study documents intergenerational shifts and varying heteroplasmy levels among oocytes and F1 offspring of New Zealand chinook salmon. Detected deviations in allele frequencies between mother and both F1 offspring and oocytes confirms the assumption that mtDNA is exposed to a genetic bottleneck or bottleneck effect during early developmental stages and are thus congruent with previous findings in other species (Hauswirth and Laipis 1982; Solignac et al. 1984; Rand and Harrison 1986; Ashley et al. 1989; Koehler et al. 1991; Howell et al. 1992; Bendall et al. 1996; Jenuth et al. 1996; Cao et al. 2007; Cree et al. 2008; Stewart et al. 2008).

However, the timing and strength of this bottleneck remains a point of uncertainty and contention. To examine whether this bottleneck occurs during oogenesis, embryogenesis, or both, we compared changes in allele frequency between mature oocytes and F1 offspring of heteroplasmic females and estimated the number of segregating units between both mother/oocyte pairs and mother/offspring pairs. The number of segregating units was estimated to be 109.3 for oogenesis (N_eOog) and 105.4 for embryogenesis (N_eEmb). Although N_eOog has been estimated to be slightly higher than N_eEmb, differences in allele frequencies for the mutant allele detected in F1 offspring and oocytes were not significantly different. As estimates of N_e are directly correlated to measurement error, the detected difference in N_e may be due to a higher measurement error for oocytes (2.12%) compared to that for F1 offspring (1.11%). Why pyroseqencing reactions of oocyte samples reveal a higher measurement error is unclear, but a possible explanation might be the presence of higher amounts of residual detergents in DNA extracts necessary for DNA extraction from oocyte samples. Such contaminations can potentially impact on downstream applications, such as pyrosequencing. Furthermore, random sample choice

could have introduced a non-significantly bias towards samples with lower variation in allele frequency, causing a higher N_eOog.

Nonetheless, N_eOog and N_eEmb, as estimated in this study, show a high degree of similarity. Thus, our results strongly suggest that the major genetic bottleneck or bottleneck effect causing heteroplasmy shifts among F1 offspring occurs during oogenesis and not embryogenesis. As allele frequencies of oocyte and F1 offspring samples are not significantly different, unequal partitioning of mtDNA during early stages of embryogenesis does not seem to contribute significantly to segregation of mtDNA haplotypes between generations. Rather, our results strongly support previous findings (Jenuth et al. 1996) that segregation of mtDNA haplotypes occurs during oogenesis and appears to be complete at the end of oocyte maturation.

As proposed previously, possible mechanisms causing the documented heteroplasmy shifts in oocytes are relaxed replication of mtDNA and random partitioning of mitochondria (Jenuth et al. 1996). These mechanisms typically depend on vast cell proliferation and mtDNA replication, two processes that can be observed during germline development. Jenuth and co-workers (1996) further proposed that the process of segregation is likely to occur during the differentiation of PGCs to oogonia and oogonia to primary oocytes as detected haplotype variations among immature and mature oocytes in their study were not significantly different.

Our findings contradict those, recently published by Cree and co-workers (2008), who report that mtDNA segregation during early embryogenesis is sufficient to induce detected heteroplasmy shifts observed among offspring in a strain of heteroplasmic mice. Admittedly, one needs to be cautious when comparing their findings with ours, considering that the model organisms used in these two studies are members of different vertebrate groups. Taxa-specific differences, such as the 10,000-fold higher mtDNA content of

chinook salmon oocytes (compared to mammalian oocytes) or differences in cleavage patterns (rotational holoblastic in mammals vs. discoidal meroblastic in teleosts) could potentially impact on the size, mechanisms and occurrence of mtDNA bottlenecks (Gilbert and Singer 2006; Santos et al. 2006).

On the other hand, previous studies suggest that the size of the intergenerational mtDNA bottleneck is unexpectedly similar across a wide range of different taxa, spanning invertebrates to vertebrates, supporting the idea of a conserved mechanism which might be comparable between taxa (Solignac et al. 1984; Rand and Harrison 1986; Ashley et al. 1989; Howell et al. 1992; Bendall et al. 1996; Jenuth et al. 1996). Furthermore, despite the 10,000-fold higher mtDNA content of chinook salmon oocytes, the estimate for N_eOog and N_eEmb in this study does not correlate with mtDNA load as our estimates are in concordance with those in mammals. Finally, our results are also in concordance with earlier studies, investigating the transmission of heteroplasmy in mice, strongly supporting that the mtDNA bottleneck occurs during oogenensis (Jenuth et al. 1996; Fan et al. 2008).

How can the incongruity between these studies be explained? It should be noted that Cree and co-workers (2008) reached their conclusion partly based on mathematical modeling, rather than directly measuring the change in haplotype frequency at different stages of both oogenesis and embryogenesis. Cree and co-workers (2008) measured the mtDNA content of single cells at different developmental stages during both oogenesis and embryogenesis to monitor changes in mtDNA content and used these data partly to modify and re-fine a mathematical model simulating the intergenerational segregation of mtDNA. Furthermore, actual measurements of mtDNA content per cell were used to confirm that the model considered the correct mtDNA content per cell at different stages of the simulation. Cree and co-workers (2008) then determined the change in allele frequency among litters of heteroplasmic mice and compared these results with results they gained by

predicting the change in allele frequencies with their newly modified model. Mathematic predictions of this model where in agreement with actual heteroplasmy measurements (91% of predicted variants were within 95% confidence interval), which was regarded as the confirmation of the model`s utility (Cree et al. 2008). According to this model, the authors concluded that 70% of the detected heteroplasmy variance among F1 offspring is generated by vegetative segregation of mtDNA during embryogenesis and 30% developed during PGC proliferation during early stages of oogenesis (Cree et al. 2008). The observed agreement between mathematical predictions and actual measurement is indeed impressive, but we find that if such results (from indirect analysis) strongly contradict results from direct analysis (Jenuth et al. 1996), these results should be further examined to remove the spectre of doubt. As Cree and co-workers (2008) had single cells and tissue from all relevant stages of both oogenesis and embryogenesis at their disposal, it would have been desirable if the change in allele-frequency, and not just the mtDNA content, would have been measured in a limited number of cells at each of these stages. This would have allowed for further improvement in their model and enabled an examination of the changes in allele frequencies during all developmental stages to accurately pinpoint the bottleneck.

In conclusion, our results support the idea of a bottleneck effect during early developmental stages of chinook salmon. Estimates for this bottleneck in this study are in concordance with estimates in other species, indicating that mechanisms around mitochondrial inheritance may be conserved and of a comparable nature among different taxa.

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Chapter 7

7 General Summary and Conclusion

7.1 Overview

Recent revelations of paternal leakage and heterologous recombination in the animal kingdom (Chapter 1, Table 1.1) challenge our current knowledge about mtDNA inheritance and the utility of mtDNA as a molecular marker. Both events potentially introduce new haplotypes into populations and therefore impact on the interpretation of mtDNA analysis (Eyre-Walker 2000; Schierup and Hein 2000; Slate and Gemmell 2004). To date, it is unclear whether the documented cases of either recombination or paternal leakage are exceptions to the general rule or if these events occur more frequently than so far believed. If either event occurred at a measurable frequency, it is vital to implement such data into models of mtDNA evolution to improve the accuracy at which evolutionary relationships and times of divergence are estimated.

In this thesis, I sought to determine the frequency of paternal leakage in chinook salmon (Chapter 3) and further investigated two major mechanism which are believed to play key roles to prevent paternal leakage: The dilution of paternal mtDNA by maternal mtDNA upon fertilization (Ankel-Simons and Cummins 1996; Chapters 4 and 5) and the presence of a genetic bottleneck during early developmental stages (Bergstrom and Pritchard 1998; Jansen and de Boer 1998; Chapter 6). Results gained in this thesis enable me to determine whether paternal leakage in salmon is likely to be a frequent or rare phenomenon, to potentially infer its frequency and to evaluate the accumulating evidence of paternal leakage in recent years and its importance for mtDNA analysis. The use of chinook salmon as a model organism to follow mitochondrial inheritance and to screen for paternal leakage is sensible as paternal leakage and subsequently recombination have been detected in salmon (Ciborowski 2007).

7.2 Screening for paternal leakage

To elucidate whether the documented cases of paternal leakage are exceptions to the general rule or repeated events occurring at low frequencies, I screened offspring of New Zealand chinook salmon for the presence of paternally derived mtDNA (Chapter 3). Offspring were generated between individuals harboring distinct mtDNA haplotypes, enabling me to unambiguously distinguish between maternal and paternal mtDNA. The experimental approach for the detection of paternal mtDNA was specifically developed for this objective to ensure highest possible sensitivity combined with the ability to process large sample sizes (Wolff and Gemmell 2008, Chapter 2). A total of 10,082 samples were screened and no paternal mtDNA was detected. According to the sample size of single crossings, paternal leakage was excluded to occur at frequencies higher than 0.18% to 0.84% (power of 0.95) and 0.27% to 1.28% (power of 0.99). The failure to detect paternal leakage in a sample size of roughly 10,000 offspring clearly indicates that the intergenerational transmission of paternal mtDNA in salmon appears to be an infrequent and rare phenomenon.

As discussed in Chapter 3, the majority of detected cases of paternal leakage (12 out of 15) occurred in hybrid zones. Why are there more documented incidents in hybrid zones? A rather simple approach to answer this question is to consider sequence differences between paternal and maternal mtDNA. Sequence differences between species are expected to be higher than within a species, potentially facilitating the detection of paternal leakage in hybrid zones. If this is indeed the case, the difference in documented incidents between hybrid and intraspecific settings could be simply due to a bias introduced by the ease of detection of inter-species sequence differences. It should also be noted that some studies documenting paternal leakage effectively took advantage of such sequence differences and actively generated hybrids or worked on existing unnatural

hybrid populations (for research or agricultural purposes) to monitor mtDNA inheritance (Gyllensten et al. 1991; Meusel and Moritz 1993; Shitara et al. 1998; Arunkumar et al. 2006; Guo et al. 2006; Sherengul et al. 2006). The manipulation and subsequent examination of such populations clearly introduces further bias towards the detection of paternal leakage in hybrids.

However, apart from such biases, fertilization experiments in Drosphila and cattle have indeed demonstrated that paternal leakage occurred at significantly higher rates in interspecific environments than in intraspecific environments (Sutovsky et al. 2000; Sherengul et al. 2006). Sutovsky and co-workers (2000) demonstrated that the active proteolytic destruction of sperm mitochondria, which is believed to add to the mechanisms preventing paternal leakage, failed in hybrids of domestic cow and wild cattle. As possible explanation they argue that species-specific sequence differences potentially lead to differences in amino acid sequence of single components of the proteolytic apparatus (Sutovsky et al. 2000). Consequently, single components of this process may not be compatible among species, and thus fail to recognize and recycle foreign sperm mitochondria. If the fate of paternal mtDNA, whether it will be inherited to offspring or not, was limited by just the process of proteolytic destruction, these findings could potentially explain why there are more documented cases of paternal leakage among hybrids than in natural populations. However, as additional mechanisms to prevent paternal leakage exist and evidence for ubiquitination of mitochondria is currently limited to mammalian species, it would be short-sighted to link paternal leakage exclusively to the success and failure of proteolytic destruction. On the contrary, if we considered active degradation of sperm mitochondria to be present in all mammals, then, evidence for paternal leakage in humans clearly questions the efficiency of such process (Schwartz and Vissing 2002). Furthermore, the lysis of mitochondria does not necessarily result in the

degradation of mtDNA contained in these mitochondria. Instead, given that mitochondria are capable of unspecific DNA import (Szabo et al. 1998; Koulintchenko et al. 2003), it is conceivable that paternal mtDNA (once released upon lysis of paternal mitochondria) can be taken up by maternal mitochondria and subsequently be inherited.

However, to understand better the occurrence of paternal leakage and the mechanisms leading to the exclusion of paternal mtDNA from inheritance, it is desirable to extend studies on the ubiquitination of sperm mitochondria to taxa other than mammals. Such work would not only help to elucidate whether this process is specific to mammals or present across different taxa but would also evaluate the process of ubiquitination as a mechanism to prevent paternal leakage. If restricted to mammals, it is likely that the ubiquitination of sperm mitochondria serves simply as an additional mechanism which acts in concert with less species-specific and more conserved mechanisms to effectively prohibit the transmission of paternal mtDNA. If this is indeed the case, such a finding would further indicate the presence of species- or taxa-specific mechanisms which are most likely to impact on the probability of paternal leakage within such groups.

7.3 The dilution effect in zygotes

It is generally accepted that the dilution of paternal mtDNA by maternal mtDNA in the zygote contributes to the main mechanisms preventing paternal leakage (Birky 1995; Ankel-Simons and Cummins 1996). The many-fold dilution of paternal mtDNA does not exclude paternal mtDNA from inheritance *per se*, but it clearly impacts on the probability at which paternal mtDNA is inherited. As explained in Chapter 5, only a subset of mitochondria, and therefore only a subset of mtDNAs, present in zygotes ultimately contribute to the arising germ layers and subsequently to the developing embryo. If this

sampling of just a fraction of mitochondria in the developing embryo is a random process, then, the probability at which paternal mtDNA is inherited to offspring is directly correlated to the ratio of paternal and maternal mtDNA in the zygote. To date, estimates for the dilution effect are limited to two mammalian species, human and mouse. This ratio has been estimated to be between $1:5.68x10^5$ and $1: 2.84x10^4$ in human zygotes and between $1:1.6x10^4$ and $1:2.1x10^3$ in mouse zygotes (Hecht et al. 1984; Shitara et al. 2000; Steuerwald et al. 2000; Reynier et al. 2001; Barritt et al. 2002; May-Panloup et al. 2003; Chan et al. 2005; May-Panloup et al. 2005; Santos et al. 2006; Amaral et al. 2007).

In this thesis, I extended such estimates to the first non-mammalian vertebrate, a teleost. I quantified the mtDNA content of both female and male gametes. I estimated the mtDNA content to be 5.73 ± 2.28 mtDNA molecules per male gamete (Chapter 4, Table 4.3) and $3.15x10^9 \pm 9.98x10^8$ mtDNA molecules per female gamete (Chapter 5, Table 5.2). As discussed in Chapter 5, the observed mtDNA content of salmon spermatozoa is comparable to that of mammalian species, despite significant differences in duration of sperm motility and mitochondrial organization and complexity. Such similar mtDNA content among highly divergent vertebrates may indicate the reduction of mtDNA being a key factor of spermatogenesis to ensure mitochondrial functionality on the one hand and to avoid paternal leakage on the other hand. In contrast to male gametes, the observed difference in mtDNA load between mammalian and teleost oocytes is significant. The mtDNA content of salmon oocytes is approximately 4-5 orders of magnitude higher compared to mammalian oocytes. As a result, the ratio of paternal to maternal mtDNA in salmon zygotes is estimated to be $1:7.35x10^8 \pm 4.67x10^8$, approximately 3 to 5 orders of magnitude smaller than the ratio revealed for mammals. Consequently, if paternal leakage was directly linked with the dilution of paternal mtDNA upon fertilization, paternal inheritance of mtDNA may be more likely per individual in mammals than in salmon.

Generally, this result strongly suggests that the varying strength of the dilution effect is likely to be associated to taxa-specific life traits such as gamete investment, morphology and reproductive strategy (internal vs. external fertilization) of female gametes. If this is indeed the case and if the dilution of paternal mtDNA is one of the main mechanisms to prevent paternal leakage, the mtDNA load of female gametes could potentially serve as indicator for taxa which may be more prone to paternal leakage than others. As estimates of mtDNA load are available for only a limited number of species, further studies on mtDNA content of both female and male gametes among highly divergent taxa are necessary to examine the suggested association between female and male mtDNA content and the potential occurrence of paternal leakage.

7.4 The mtDNA bottleneck

The probability at which the diminutive contribution of paternal mtDNA (after dilution in the zygote) is further inherited to offspring is dependant on the size of the genetic bottleneck acting on mtDNA during early developmental stages. To examine the size of this bottleneck and to elucidate whether this bottleneck is likely to occur either during oogenesis or embryogenesis I examined changes of allele frequency among mother/oocyte and mother/offspring pairs of heteroplasmic individuals. The number of segregating units was estimated to be 109.3 (median = 109.3; 62.4 < N_eOog < 189.6; 95% confidence interval) for oogenesis (N_eOog) and 105.4 (median = 105.4; 70.3 < N_eEmb < 153.1; 95% confidence interval) for embryogenesis (N_eEmb). Changes in allele frequency between oocyte and offspring samples were not significantly different, strongly suggesting that segregation of mtDNA occurs during oogenesis.

Considering that N_eEmb has been estimated to be 20 to 163 in Holstein cows (discussed in Rand and Harrison 1986; Ashley et al. 1989), 87 to 395 in crickets (Rand and Harrison 1986), 370 to 740 in fruit flies (Solignac et al. 1984), 2 to 349 in humans (Howell et al. 1992; Bendall et al. 1996; Jenuth et al. 1996) and 185 in mice (Jenuth et al. 1996), the size of the mtDNA bottleneck among different species and taxa appears to be similar, indicating that the mechanisms causing the bottleneck effect may be conserved. However, it is important to note that estimates of N_eEmb or $N_e$oog in single studies were generated using different mathematical approaches. The intricacies of a given mathematical model and changes among parameters are likely to impact on the outcome of such studies. Therefore, it may be worth pursuing to generate new estimates by applying the same mathematical model using the data from single studies. Such reanalysis would not only improve the credibility of such comparisons, but also allow for further speculation about whether the mtDNA bottleneck is likely to be a conserved mechanism or not. Nonetheless, it should be noted that despite the significant differences in mtDNA load between mammals and salmon (as discussed in Chapters 4 and 5), the similarity between estimates for N_eEmb in both systems remains apparent and does not appear to correlate with the mtDNA load of maternal gametes.

However, if we accept the estimate of N_eEmb in this thesis to be accurate, it is possible to approximate the probability at which paternal mtDNA is inherited to offspring in salmon. Considering that 105.4 (median = 105.4; $70.3 < N_e\text{Emb} < 153.1$; 95% confidence interval) mtDNAs are transmitted to offspring and that the ratio of paternal to maternal mtDNA is estimated to be $1{:}7.35\text{x}10^{8} \pm 4.67\text{x}10^{8}$, the probability of paternal mtDNA being inherited to offspring is about $1.29\text{x}10^{-11}$. In other words, if the inheritance of paternal leakage was a random process and exclusively determined by the dilution and genetic bottleneck acting on paternal mtDNA, one would expect to find one case of

paternal leakage within a sample size of roughly $7.75x10^{10}$, clearly indicating the rare nature of this event.

7.5 General conclusion and future work

Accumulating evidence for paternal leakage in the animal kingdom is changing our view of mtDNA inheritance. The increasing number of documented cases is potentially due to advances in technology, such as (i) increased sensitivity for minor allele contributions, (ii) the capability of single techniques to process large sample sizes, (iii) increased generation of sequence data and therefore (iv) increased gene coverage. In combination, such advances allow for the detection of even rare phenomena, such as paternal leakage. However, given this clear evidence, it is inevitable to acknowledge the occurrence of paternal leakage in the animal kingdom in both intraspecific and interspecific environments. More importantly, the potential incidence of this phenomenon and its influence on subsequent analyses must be considered if mtDNA is applied as a molecular marker. The question which needs to be answered now is no longer about the existence of paternal leakage but about its frequency and if such frequencies can be implemented into models of mtDNA evolution.

As presented in this thesis, the dilution of paternal by maternal mtDNA upon fertilization and the genetic bottleneck acting on mtDNA during oogenesis are most likely key factors preventing paternal leakage. If this is indeed the case and if paternal leakage was a purely stochastic process, it would be possible to infer theoretical frequencies of this phenomenon based on the ratio of paternal to maternal mtDNA in zygotes and the number of segregating units between generations. Such frequencies could then potentially be implemented into models of mtDNA evolution to improve the accuracy of such models.

However, as the ratio of paternal to maternal mtDNA in zygotes has been documented to vary significantly and the size of N_eEmb to vary moderately, species- or taxa-pecific variations of these mechanisms need to be considered and incorporated into such models. Also, most importantly, the presence of additional species- or taxa-specific mechanisms, such as the ubiquitination of sperm mitochondria (Sutovsky et al. 1999) or the simple exclusion of sperm mitochondria from inheritance *per se* (Ursprung and Schabtach 1965), must be considered and accounted for. Unfortunately and as matter of fact, such specific processes are not random and potential failure rates are difficult to determine. Therefore, it appears intricate to infer accurate theoretical frequencies for any species, revealing any kind of species-specific mechanism to prevent paternal leakage. Nevertheless, in those species for which a purely random process of mtDNA inheritance can be confirmed, a theoretical frequency can be inferred and implemented into models of mtDNA evolution. To achieve this, further studies need to be undertaken to (i) determine N_eEmb among highly divergent taxa, (ii) determine the ratio of paternal to maternal mtDNA in zygotes among different taxa, (iii) clarify if the ubiqitination of sperm mitochondria is restricted to the mammalian system, and to (iv) identify additional species- or taxa-specific mechanism that potentially prevent further paternal leakage.

Furthermore, as the dilution of paternal mtDNA upon fertilization appears to be a major factor to prevent paternal leakage, it is important to determine the dilution effect among highly divergent taxa. As indicated in this thesis, the mtDNA content of sperm may be conserved. Ocytes instead appear to vary in their mtDNA content significantly, most likely reflecting the metabolic demands of the developing embryo (*e.g.* internal vs external development). Nonetheless, it must also be noted that differences in mtDNA load between mammals and salmon may simply reflect the difference in oocyte dimension. Further, because mitochondrial activity is associated with heat production, it is also possible that

the number of mitochondria per oocyte may vary according to environmental factors to maintain crucial temperatures for successful development during embryogenesis. In such case, salmon oocytes developing on the bottom of a cold stream potentially rely on a much higher number of mitochondria compared to oocytes of animals that develop in milder and/or dry conditions. However, the description of the dilution effect with particular emphasis on oocytes among highly divergent taxa and in regard to different climate zones, different environments and oocyte size may offer a great opportunity to further understand details of mitochondrial inheritance and could potentially identify species or taxa which may be prone to paternal leakage (i.e., species low in mtDNA oocyte content) by the dilution effect only.

Also, major areas of future work in which to investigate further details and mechanisms of mtDNA inheritance are those in which a failure of mechanisms preventing paternal leakage has been documented predominantly: hybrid zones. Whereas the occurrence of paternal leakage among closely related individuals within populations appears to be exceptional, genetically more distinct individuals seem to be affected by this phenomenon more often. It is unclear, and the subject of much debate, whether this may be due to sequence dissimilarities between nuclear or mitochondrial genomes, or both.

Doubly uniparental inheritance (DUI) in some bivalves, for example, in which both parental mitochondrial genomes are inherited (with the paternal haplotype contributing exclusively to the male gonads) clearly indicate a potential influence of mtDNA haplotype on mtDNA inheritance and/or segregation (Breton 2007). Another factor influencing mtDNA inheritance is temperature. Temperature-dependent inheritance of different mtDNA haplotypes is known to occur in Drosophila (Matsuura et al. 1993). Proliferation and transmission of mtDNA haplotype has been shown to be dependent on incubation temperature and nuclear background. To date, the exact mechanisms of such selective

mtDNA inheritance and/or segregation are unknown and further work is needed to unveil their secrets and their potential role.

7.6 References

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