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ORIGINAL ARTICLE

Lost in the zygote: the dilution of paternal mtDNA upon fertilization

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The mechanisms by which paternal inheritance of mitochondrial DNA (mtDNA) (paternal leakage) and, subsequently, recombination of mtDNA are prevented vary in a species-specific manner with one mechanism in common: paternally 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 chinook 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 chinook salmon oocytes to be $3.2 \times 10^9 \pm 1.0 \times 10^9$, 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 $1{:}5.5 \times 10^8$. This contribution of paternal mtDNA to the overall mtDNA pool in salmon zygotes is three to five 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.
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Introduction

Animal mitochondrial DNA (mtDNA) is the marker of choice for a wide range of applications, such as phylogeography, phylogenetics and population genetics (Avise *et al*., 1987; Moritz *et al*., 1987; Avise, 2004). Central to its success are several key characteristics, such as its high copy number, small size (15–20 kb), higher mutation rate (compared with 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 (Avise, 2004; 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 (Ladoukakis and Zouros, 2001), amphibians (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 nematodes (Lunt and Hyman, 1997; Armstrong *et al*., 2007). What is more, gender-associated and tissue-specific transmission of both paternal and maternal mtDNA ('doubly uniparental inheritance' (DUI)) appears to be the norm in some bivalves (Breton *et al*., 2007). Although not the general rule, the increasing number of documented cases for paternal leakage and recombination of mtDNA 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). In tunicates, for example, sperm mitochondria do not enter the egg, whereas in mammals, sperm are actively tagged with ubiquitin leading to proteolytic digestion by the female cell (Sutovsky, 2003). The effectiveness and reliability of such mechanisms at preventing paternal mtDNA transmission are not fully understood (Birky, 2001; Thompson *et al*., 2003), and may fail at times (Schwartz and Vissing, 2002). However, if such mechanisms fail, the inheritance of paternal mtDNA is further inhibited by a mechanism that appears to apply to most species: 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 is defined by the mtDNA content of the parental gametes. However, this ratio does not necessarily reflect the

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extent of paternal leakage in the developing embryo. The mtDNA content remains constant during early embryogenesis (Cao *et al*., 2007; Cree *et al*., 2008) and mitochondria are apportioned to arising cells in a random fashion. Because most of these cells form extraembryonic tissues (Hogan *et al*., 1986), only a subset of all cells and consequently mtDNAs present in the zygote will ultimately contribute to 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 at which the paternal mtDNA will contribute to the subset of cells giving rise to the actual embryo. Consequently, the quantification of mtDNA in gametes, that is 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 10–75 and 1.6×10^5 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 1.4–6.8 molecules (May-Panloup *et al*., 2003; Amaral *et al*., 2007) and oocytes 1.93–7.95×10^5 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 $1{:}5.7 \times 10^5$–$1{:}2.8 \times 10^4$ of paternal-to-maternal mtDNA in human and $1{:}1.6 \times 10^4$–$1{:}2.1 \times 10^3$ 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 the mtDNA content of gametes in a systematic way (that is, within different taxa) could provide valuable insights into the broader patterns of mtDNA inheritance.

Here, we determine the mtDNA content of chinook salmon oocytes and build on earlier work examining the mtDNA content of sperm (Wolff and Gemmell, 2008), 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.

Materials and methods

Oocyte samples

Samples were collected during the spawning season in April and May 2005 from a hatchery population of chinook salmon (*Oncorhynchus tshawytscha*) in collaboration with the National Institute of Water and Atmospheric Research (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 −20 °C until analysis.

Preparation of DNA

DNA was extracted from whole eggs in 1.5 ml extraction buffer (final volume including a single egg), containing 4 M urea, 1% Tween 20, 1% Nonidet P-40, 5% Chelex-100 and Proteinase K ($0.4\,\mu g\,\mu l^{-1}$) (Aranishi, 2006). To improve the digestion, eggs were disrupted using a sterile glass rod. Samples were then incubated using a thermal shaker for 6 h at 55 °C (shaking intervals of 5 s at 1400 r.p.m. every 10 min). After incubation, samples were boiled for 8 min, centrifuged for 2 min at 20 000 *g*, diluted 1:40 in TE (10 mM Tris, 1 mM EDTA, pH 8) and stored at −20 °C. DNA extracts were not further purified to avoid the 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 twofold serial dilutions.

Construction of the external standard (reference DNA)

To quantify the number of mitochondrial genomes per oocyte by real-time PCR, we amplified a 314-bp fragment of the mitochondrial NADH dehydrogenase subunit 1 gene (*mt-nd1*) as an external standard (Bustin, 2004). PCR reactions were carried out with the forward primer ND1aF (5′-GGTAATTGCGAGAGGCCTAA-3′) and the reverse primer ND1R1 (5′-GTAAGGGCAAGTATGGGT GT-3′) under standard conditions with 25 ng of whole genomic DNA in 25 µl reactions (primers according to sequence information of the mitochondrial genome of *O. tshawytscha* (NC_002980) available at the National Institute of Biotechnology Information (NCBI)). PCR amplifications consisted of 2-min initial denaturation and 30 cycles of denaturation at 94 °C for 15 s, annealing at 57 °C for 20 s and elongation at 72 °C for 20 s.

The PCR product was cloned using a GeneJet PCR cloning kit (Fermentas, Burlington, Ontario, Canada), and the recombinant plasmid was purified using a PureLink 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 an 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 1 µg of purified DNA was estimated to contain 2.7×10^{11} molecules (http://www.molbiol.edu.ru/eng/scripts/h01_07.html). Tenfold serial dilutions (10^2 copies per µl to 10^7 copies per µl) were prepared, aliquoted and stored at −20 °C.

Quantification of the number of mtDNA molecules per oocyte

DNA extracts from oocyte samples were analyzed using an 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 20 µl reaction volumes, containing 1 × Fast Start SYBR Green Master mix, 1 µl DNA and 10 pmol of each primer. Quantitative polymerase chain reaction (Q-PCR) amplifications consisted of initial denaturation for 10 min at 95 °C, followed by 36 cycles with 20 s denaturation at 95 °C, annealing for 20 s at 57 °C and elongation at 72 °C for 20 s. 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 nonspecific amplification. In addition, as nuclear-encoded mitochondrial pseudogenes may affect our estimates, we performed BLAST searches using the mtDNA sequence that we quantified in our study against salmonid databases (http://www.ncbi.nlm.nih.gov/blast/) to reveal the potential presence of such genes in chinook salmon.

Amplifications of the reference DNA (10-fold serial dilutions of the recombinant plasmid, 10^2–10^7 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 (40 ×) and the initial amount of extraction buffer (1500 ×). Therefore, results from Q-PCR experiments were corrected by 60 000 for the final estimate of mtDNAs per oocyte.

Results

Applicability of the Q-PCR assay

Amplifications of the external standard (Figure 1a) and serial dilutions of sample DNA (Figure 1c) were performed with efficiencies of 99.6 and 99.3%, respectively. Slopes of derived standard curves were −3.332 for the external standard (Figure 1b) and −3.333 for sample DNA (Figure 1d). As a measure of accuracy, the high similarities of slopes and efficiencies between standard and sample DNA confirmed the applicability of this Q-PCR assay to estimate the mtDNA content of oocytes.

To control for the absence of nuclear-encoded mitochondrial pseudogenes, which could potentially influence our measurements, we BLASTed the mtDNA sequence that we quantified in our study against salmonid databases. This search did not detect any similar sequences other than mitochondrial *mt-nd1* sequences (data not shown). Melting curve analyses at the end of each quantification experiment further confirmed the amplification of one single sequence in all experiments, excluding the co-amplification of nuclear-encoded mitochondrial pseudogenes of different length or nucleotide composition (data not shown).

Reproducibility

To assess the reproducibility of mtDNA quantifications, three oocytes with varying mtDNA contents were analyzed 10 times. Amplifications of these samples revealed highly repeatable amplification curves and, therefore, highly repeatable estimates for the mtDNA content. Estimates for the number of mtDNAs per oocyte varied between 2.3×10^9 and 3.2×10^9 for three different oocytes, and the coefficient of variance (CV) was 4.8% for oocyte one, 1.8 and 1.6% for oocytes two and three, respectively (Table 1). Low CVs confirmed the utility of the Q-PCR assay and the highly reproducible nature of the experimental measures.

Oocyte mtDNA content

This Q-PCR assay was applied to measure the mtDNA content in oocytes from 17 different individuals. For each

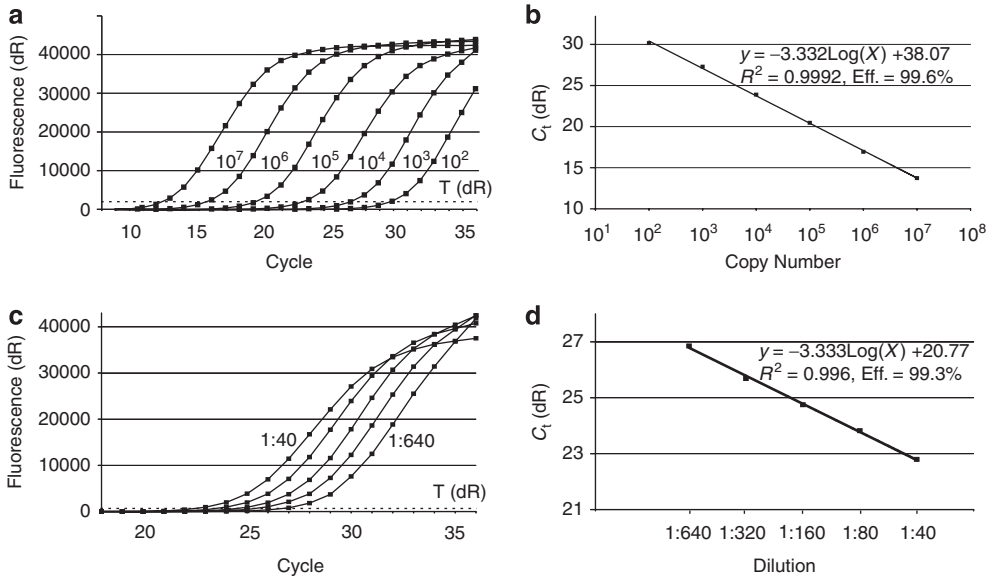

Figure 1 Amplification plots and standard curves of the external standard and sample DNA; C_t(dR), indicates threshold cycle; T(dR), indicates threshold fluorescence. (**a**) Amplification plots of the external standard (10-fold dilution series); relative fluorescence (dR) plotted against cycle number. (**b**) Standard curve derived from amplification plots of external standard; threshold cycle C_t(dR) plotted against initial amount of template (copy number). (**c**) Amplification plots of sample DNA (twofold dilution series); relative fluorescence (dR) plotted against cycle number. (**d**) Standard curve derived from amplification plots of sample DNA; threshold cycle C_t(dR) plotted against dilution step.

Table 1 Intra-assay variance in mtDNA load per oocyte ($\times 10^9$)

Oocyte	*1*	*2*	*3*
	2.2	2.9	3.3
	2.4	2.9	3.2
	2.4	2.9	3.3
	2.2	3.0	3.3
	2.4	3.0	3.2
	2.4	3.0	3.2
	2.1	3.0	3.2
	2.3	2.9	3.3
	2.2	2.9	3.2
	2.3	3.0	3.2
Mean	2.3	3.0	3.2
s.d.	0.11	0.05	0.05
CV (%)[a]	4.8	1.8	1.6

Abbreviation: mtDNA, mitochondrial DNA.
[a]Coefficient of variance with CV = s.d./mean.

Table 2 Mean mtDNA load of oocytes per individual

Individual	*Mean for five oocytes per individual*			*Inter-oocyte variation*
	mtDNA content ($\times 10^9$)	*s.d.* ($\times 10^8$)	CV_{av} (%)[a]	CV_{i-o} (%)[b]
201	2.8	0.9	3.3	7.7
208	1.8	0.6	3.0	10.7
209	3.4	0.9	2.7	5.5
213	2.3	1.1	4.6	4.5
214	1.4	0.8	5.4	13.4
227	3.8	1.3	3.6	5.3
239	3.9	1.7	4.2	9.6
243	1.1	0.4	3.7	18.4
254	3.8	1.6	4.2	3.5
256	4.1	1.8	4.5	14.0
263	2.9	1.0	3.4	4.5
268	2.9	1.3	4.4	2.5
275	3.2	1.5	5.0	14.6
280	4.0	1.3	3.5	6.6
283	2.2	0.8	3.6	3.4
285	3.1	1.2	3.8	4.4
357	7.0	4.0	5.6	8.0
Mean	$3.2 \pm 1.0 \times 10^9$	1.3	4.0	8.0

Abbreviation: mtDNA, mitochondrial DNA.
The s.d. of the mean mtDNA content of chinook salmon oocytes ($\pm 1.0 \times 10^9$) was calculated based on triplicate measurements of 85 oocytes (five per individual, data not shown).
CV = s.d./mean.
[a]Average coefficient of variance for five oocytes per individual (mean measurement error per individual; data not shown).
[b]Overall coefficient of variance among five oocytes per individual (variability of mtDNA content per individual, data not shown).

individual, five oocytes were analyzed, each in triplicate. The mtDNA content of oocytes per individual was calculated by the mean mtDNA content of five oocytes. The overall mean mtDNA content of 85 oocytes was $3.2 \times 10^9 \pm 1.0 \times 10^9$ molecules with an average CV of 4.0% (Table 2).

The mitochondrial DNA content among different oocytes from the same individual varied moderately with the highest variation of 18.4% for individual 243 and the lowest variation of 2.5% for individual 268 (Table 2). The overall mean inter-oocyte variation within an individual was 8.0% (Table 2). The lowest detected mtDNA load was 9.4×10^8 molecules per oocyte and the highest level was 7.7×10^9 molecules per oocyte, leading to an overall coefficient of variance among all 85 oocytes examined of 43.5% (data not shown).

Discussion

This study found the average mtDNA content of oocytes to be $3.2 \times 10^9 \pm 1.0 \times 10^9$ among 17 individuals of New Zealand chinook salmon. With an average of 3.67×10^5 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, b; 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. This finding is in agreement with stereological analyses estimating the number of mitochondria in toad and frog oocytes, indicating the mitochondrial DNA content of external fertilizers to exceed that of mammalian oocytes by at least three orders of magnitude (Romek and Krzysztofowicz, 2005; Shoubridge and Wai, 2007).

The influence of nuclear-encoded mitochondrial pseudogenes on our analysis was considered to be inconsequential as no such genes have been documented in teleosts to date (Venkatesh *et al*., 2006). However, if present in chinook salmon, such genes are unlikely to be present in high copy numbers and, therefore, would not have significantly impacted on our estimates. Moreover, as most nuclear-encoded mitochondrial pseudogenes exhibit length polymorphisms (Tourmen *et al*., 2002; Woischnik and Moraes, 2002), we excluded the presence of such genes by performing a melting curve analysis at the end of each experiment.

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 within the female reproductive tract (Gardner *et al*., 2002; Dumollard *et al*., 2007a). These nutrients are directly utilized by the developing embryo to satisfy its metabolic requirements, allowing for limited oxidative activity of mitochondria (Leese, 2002; Houghton and Leese, 2004; Dumollard *et al*., 2007b). 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 are 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 with 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. In addition to developmental distinctions, the mtDNA content of oocytes is also likely to be linked to oocyte dimension. With mammalian oocytes measuring generally between 0.07 and 0.12 mm and New Zealand chinook salmon oocytes between 4.5 and 5.5 mm in diameter, the detected difference in mtDNA content

between the two systems appears reasonable (Griffin *et al*., 2006).

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 (Wolff and Gemmell, 2008), and here, we estimate the average mtDNA content of salmon oocytes to be $3.2 \times 10^9 \pm 1.0 \times 10^9$. 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 $1{:}5.5 \times 10^8$ (quotient of mean mtDNA content per sperm and mean mtDNA content of oocytes). Accordingly, the contribution of paternal mtDNA to the overall mtDNA pool in salmon zygotes is three to five orders of magnitude smaller compared with 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 extraembryonic tissues (Fleming *et al*., 1992; Dumollard *et al*., 2007a). Thus, a genetic bottleneck comparable to that described for mammalian embryogenesis is expected in teleosts. If species-specific mechanisms to prevent paternal leakage (Birky, 1995) break down, the dilution of paternal mtDNA by maternal mtDNA might serve as an efficient barrier to avoid the transmission of paternally derived mtDNA to offspring. Consequently, paternal inheritance of mtDNA may be more likely per individual in mammals than in salmon.

Finally, considering a comparable mtDNA content for mammalian and chinook salmon sperm (Wolff and Gemmell, 2008), it appears that the varying strength of the dilution effect between the two systems is mainly determined by the mtDNA content of the female gametes. If this relation finds general application across taxa and if the mtDNA content of oocytes is linked to oocyte size, as indicated by this work, the probability of paternal leakage occurring, based on the dilution effect only, might then be linked to oocyte size and gamete investment in general. Thus, it is desirable that further study be undertaken across a range of other taxa to test this hypothesis and to better understand the mechanisms of mitochondrial inheritance.

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