

Cytonuclear conflict in interpopulation hybrids: the role of RNA polymerase in mtDNA transcription and replication

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Abstract

Organismal fitness requires functional integration of nuclear and mitochondrial genomes. Structural and regulatory elements coevolve within lineages and several studies have found that interpopulation hybridization disrupts mitonuclear interactions. Because mitochondrial RNA polymerase (mtRPOL) plays key roles in both mitochondrial DNA (mtDNA) replication and transcription, the interaction between mtRPOL and coevolved regulatory sites in the mtDNA may be central to mitonuclear integration. Here, we generate interpopulation hybrids between divergent populations of the copepod *Tigriopus californicus* to obtain lines having different combinations of mtRPOL and mtDNA. Lines were scored for mtDNA copy number and ATP6 (mtDNA) gene expression. We find that there is a genotype-dependent negative association between mitochondrial transcriptional response and mtDNA copy number. We argue that an observed increase in mtDNA copy number and reduced mtDNA transcription in hybrids reflects the regulatory role of mtRPOL; depending on the mitonuclear genotype, hybridization may disrupt the normal balance between transcription and replication of the mitochondrial genome.

Introduction

Genetic differentiation of geographically isolated conspecific populations may arise as a consequence of mutation, genetic drift and natural selection. Subsequent interpopulation hybridization of diverged populations often results in a pattern of F₁ hybrid vigour; however, crossing of F₁ individuals to produce F₂ hybrids can result in sharp reductions in mean fitness traits as well as increased variance in those traits (Endler, 1977; Burton *et al.*, 2006). Although such a pattern of reduced hybrid fitness clearly indicates the involvement of negative epistatic interactions, the molecular mechanisms underlying F₂ hybrid breakdown in fitness are not fully understood.

One potential mechanism for hybrid breakdown involves interactions between nuclear and mitochondrial genomes. This hypothesis is based primarily on three observations: (i) mitochondrial energy production explic-

itly requires interactions between mitochondrial DNA (mtDNA) and nuclear DNA (nucDNA) genes; (ii) mtDNA typically evolves 10-fold faster than nucDNA; and (iii) populations often show sharp differentiation in mtDNA haplotypes. Combined, these observations suggest that rapid mtDNA evolution may lead to fixation of different nuclear-encoded compensatory substitutions within different natural populations (Rand, 2001). Under this model, F₂ hybrids homozygous for compensatory alleles from one population will function poorly in the presence of mtDNA from another population. Such detrimental nuclear/mitochondrial genomic interactions have been documented in diverse taxa (e.g. Lee *et al.*, 2008; Niehuis *et al.*, 2008; Arntzen *et al.*, 2009) and direct tests of the hypothesis in interpopulation crosses of the copepod *Tigriopus californicus* (Baker, 1912) strongly support the role of nucDNA–mtDNA (mitonuclear) interactions in F₂ hybrid breakdown (Ellison & Burton, 2008a).

Burton *et al.* (2006) and Gershoni *et al.* (2009) suggest that disrupted mitochondrial energy production and function may be a primary cause of F₂ hybrid breakdown. However, the mitonuclear interaction network is highly complex and breakdown may involve many

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interacting loci, potentially involved in multi-member epistatic complexes. One subset of such interactions is that involved in the gene regulatory system, disruption of which in a hybrid genetic context may result in abnormal mitochondrial gene regulation. Mitochondrial regulation is largely controlled via the nucleus. The complete suite of proteins necessary for transcription and replication of mtDNA are transcribed in the nucleus, translated in the cytoplasm and imported as polypeptides into the mitochondrion (Shadel & Clayton, 1997; Ikeda & Gray, 1999). The highly simplified, phage-derived mitochondrial transcriptional apparatus is typically comprised of two transcription factors (TFAM and TFBM) acting in concert with a single-subunit mitochondrial RNA polymerase (mtRPOL; Gaspari *et al.*, 2004). Unlike the highly multimeric nuclear RNA polymerase complexes, mtRPOL is itself responsible for promoter-binding strength and specificity (Gaspari *et al.*, 2004). Hybrid combinations of mtRPOL and mitochondrial promoter can have marked impact on mitochondrial transcription, even eliminating transcriptional activity when relatively distant mtRPOL and mitochondrial promoters are paired *in vitro* (Gaspari *et al.*, 2004).

Interestingly, mtDNA replication is also dependent on mtRPOL. Replication of mtDNA is initiated only after an RNA primer is generated by mtRPOL, then proceeds with a designated mtDNA polymerase enzyme complex (Falkenberg *et al.*, 2007). As a consequence, it is possible for defects in mtDNA transcription to have secondary impacts on mtDNA replication (Clayton, 2000). Reduced mtDNA copy number has been associated with a disparate group of pathologies having severe and often fatal consequences that are collectively referred to as mtDNA depletion syndromes (Durham *et al.*, 2005). The causes of this heterogeneous group of syndromes are generally thought to be autosomally recessive and are often associated with components of the mtDNA synthesis machinery (Naviaux & Nguyen, 2004). Although diminished mtDNA copy number appears to have strongly deleterious effects on health and fitness, increased mtDNA copy number in low motility sperm has been suggested as a beneficial mechanism to partially compensate for loss of mtDNA integrity (e.g. an excessive mitochondrial mutational load; Song *et al.*, 2005).

Cellular mtDNA content is highly variable relative to nuclear genomic DNA content. Whereas a typical eukaryotic cell contains only a single diploid copy of nuclear genomic DNA, mtDNA may have (haploid) copy numbers ranging from a few hundred to tens of thousands per cell (Bogenhagen & Clayton, 1974; Scheffler, 2008). In fact, cellular mtDNA copy number can vary between tissue types within an organism and even between individual mitochondria within a single cell (Satoh & Kuroiwa, 1991; Barazzoni *et al.*, 2000; Cavelier *et al.*, 2000). Despite this striking difference between nuclear and mitochondrial cellular genomic

content, polypeptides encoded by both genomes must interact with defined stoichiometry in the oxidative phosphorylation (OXPHOS) pathway of each cell (Krause *et al.*, 2006). Of the approximately 140 protein species comprising integral components of the OXPHOS pathway, the mitochondrial genome itself typically encodes only 13 in animals (Larsson & Clayton, 1995; Scheffler, 2008). Over 75% of cellular energy can be produced via the OXPHOS system (Rodríguez-Enríguez *et al.*, 2008); so efficient integration of nuclear- and mitochondrial-encoded components is of paramount importance to cellular viability. To achieve such integration, regulation of these 13 mitochondrial-encoded OXPHOS components must be modulated to account both for the regulatory state of nuclear-encoded OXPHOS components and for variation in mtDNA content in each cell.

Both mitochondrial OXPHOS activity and mitochondrial transcriptional activity are impacted in interpopulation hybrids of the marine copepod *T. californicus* (Baker, 1912) (Rawson & Burton, 2002; Ellison & Burton, 2006, 2008b; Harrison & Burton, 2006). Allopatric populations of *T. californicus* are highly divergent, particularly at mitochondrial loci (Burton, 1998; Burton & Lee, 1998; Edmands, 1999) and show a ubiquitous pattern of F₂ hybrid breakdown in several fitness characters (Burton, 1990; Edmands, 1999). Although disruption may involve both nuclear-mitochondrial interactions and nuclear-nuclear components imported into the mitochondria (Willett & Burton, 2001; Rawson & Burton, 2002; Ellison & Burton, 2006; Harrison & Burton, 2006; Willet, 2006), backcross studies (Edmands & Burton, 1999; Ellison & Burton, 2008a) found that nuclear-nuclear interactions failed to explain observed fitness effects; rather, nuclear-cytoplasmic interactions, likely involving mitochondria accounted for the hybrid breakdown phenotype. Subsequently, it was found that interactions between mtRPOL and mtDNA appear to have a strong impact on mitochondrial transcription in interpopulation hybrids under hypo-osmotic stress conditions, with specific combinations of mtRPOL and mtDNA having a diminished stress response capacity in hybrids (Ellison & Burton, 2008b).

Here, we examine variation in mtDNA copy number with respect to particular combinations of mtRPOL and mtDNA in interpopulation hybrids of *T. californicus* and show that those genotypes with low fitness and reduced transcriptional stress response (Ellison & Burton, 2008b) are associated with significantly elevated mtDNA content. We suggest that this may be owing to the disruption of a balance between selfish mitochondrial (i.e. mtDNA replication) and cellular (i.e. mtDNA transcription) control elements as a result of hybridization. Regardless of the detailed causal mechanism, regulatory cytonuclear conflict may represent an important component of hybrid fitness reduction.

Materials and methods

Culture of *Tigriopus californicus*

Interpopulation hybrids were generated for six pairwise crosses beginning with three populations of *T. californicus*: Santa Cruz, CA, USA (SCN: 36°57'N, 123°03'W, collected April 2006), Abalone Cove, Palos Verdes, CA, USA (AB: 33°44'N, 118°22'W, collected May 2006) and San Diego, CA, USA (SD: 32°45'N, 117°15'W collected June 2006). Stock cultures of each population were kept in beakers containing 200 mL seawater at 20 °C and fed dried *Spirulina* algae. All experimental crosses were completed in 100-mm diameter Petri dishes containing 0.1 mg ground *Spirulina* per litre filtered seawater. Animals were transferred to fresh dishes with each generation.

Recombinant hybrid inbred lines were generated as described in Ellison & Burton (2008b) and used for all measures of OXPHOS gene expression. Replicate, non-inbred crosses were prepared by placing five males and five virgin females from the desired crosses in the same dish and allowing hybridization. A noninbreeding strategy was used through the F₂ generation. Virgin F₂ females were then backcrossed to either the maternal or the paternal lineage in a similar fashion. Data in this manuscript evaluating effects of hybridization on mtDNA copy number were obtained exclusively from parental, F₁, F₂ and maternal and paternal F₂ backcross animals from these replicate crosses. Only adult animals were removed from culture for experimental work measuring mtDNA copy number.

Determination of mtDNA copy number

Copy number for mtDNA was measured in individual copepods using quantitative polymerase chain reaction (qPCR) for 10 male and 10 female adult copepods from the three parental populations, F₁ hybrids, F₂ hybrids and maternal and paternal F₂ backcrosses. DNA was prepared by digesting single copepods with 30 µL of proteinase-K cell-lysis buffer at 65 °C for 1 h followed by 85 °C for 15 min. Two 5-µL aliquots of the lysate were used to quantify ATPase c (ATPc, nuclear-encoded) and ATPase 6 (ATP6, mitochondrial-encoded) according to the protocol of Ellison & Burton (2008b).

All qPCR assays were completed on a Stratagene Mx3000P (Stratagene, La Jolla, CA, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Standards were generated using purified PCR products from parental cDNA samples quantified using a Lambda 35 UV/VIS spectrophotometer (Perkin Elmer, Waltham, MA, USA). Approximate copy number was inferred using OligoCalc (Kibbe, 2007) and standards for each target gene were diluted to include concentrations of 10¹¹, 10¹⁰, 10⁹, 10⁸ and 10⁷ copies µL⁻¹. qPCR was performed in 20-µL reaction volumes containing 5 µL of DNA, 0.25 µg mL⁻¹ of each forward and reverse

primer and 10 µL of Power SYBR Green PCR Master Mix (Applied Biosystems). Reaction conditions included a 2-min denaturing step at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 52 °C and 60 s at 72 °C. Denaturing curves were generated at the end of each series of assays to verify the specificity of the reaction. mtDNA copy number was subsequently normalized to copy number of the nuclear-encoded ATPc and is reported as a ratio of ATP6 copy number to ATPc copy number. All primers were identical to those reported in Ellison & Burton (2008b).

Genotyping of mtRPOL

mtRPOL was genotyped in F₂ hybrids, as well as maternal and paternal backcrosses, according to the protocol of Ellison & Burton (2006). DNA was prepared as described before. PCR amplification was performed with a combination of either MTRP-gtype.1F and MTRP-gtype.1R primers (for SD × AB and SD × SCN crosses) or MTRP-Msp1.F and MTRP-Msp1.R primers (for AB × SCN crosses). Amplification products were digested overnight with *Hinf*I (SD × AB and SD × SCN) or *Msp*I (AB × SCN) restriction enzymes (New England Biolabs, Cambridge, MA, USA), and scored on 2.5% agarose gels following electrophoresis. mtRPOL genotype and mtDNA copy number were determined for each F₂ hybrid, and F₂ backcross individual assayed. All primers were taken directly from Ellison & Burton (2008b).

Expression of OXPHOS-associated genes

Ellison & Burton (2008b) did not find any significant variation in expression among three mitochondrial genes: ATP6, cytochrome oxidase I, and cytochrome b, so gene expression of ATP6 was used as a representative measure of mitochondrial gene expression in this study. All gene expression data were obtained as part of this previous study with methods described therein. Briefly, gene expression data were obtained by RNA extraction and quantitative reverse-transcribed (RT) PCR from recombinant hybrid inbred lines for alpha tubulin (ATU, a nuclear-encoded housekeeping gene in this study) and the representative mitochondrial gene, ATP6. Genomic DNA was extracted from the same tissues in parallel and assayed for ATP6 to determine mtDNA copy number. Assays were completed with control animals as well as animals subjected to a hypo-osmotic stress treatment. Under this treatment, animals were transferred from 100% seawater culture conditions to 50% seawater for 30 min prior to nucleic acid extraction. The nature and duration of this stress treatment are similar to those employed in previous studies (Goolish & Burton, 1989). Normalized ATP6 expression was corrected for mtDNA copy number measured as ATP6 copy number in genomic DNA to determine expression of mitochondrial genes relative to

mtDNA dosage for all samples under all experimental treatments.

Statistical analysis

All statistical analyses were completed using SPSS 11 for Mac OSX (SPSS Inc., Chicago, IL, USA). Changes in mtDNA content and ratios of cDNA to gDNA were determined using one-way ANOVA tests with nonparametric *post hoc* Tanhane’s T2 test for pairwise comparisons ($\alpha = 0.05$). Levene’s test of homogeneity of variances was used to evaluate distribution of variances across all cross generations within each mtDNA genotype ($\alpha = 0.05$).

Results

Variation in mtDNA copy number in interpopulation hybrids

mtDNA copy number was measured as the ratio of mtDNA to genomic DNA in *T. californicus* individuals from parental lineages, F₁ and F₂ hybrids and F₂ hybrid backcrosses to both the maternal and paternal lineages in each of the six crosses. Results were grouped by mtDNA genotype and are presented in Fig. 1 and Table 1. Overall ANOVA results were significant in crosses bearing either the AB or SD mtDNA and approaching significance in those crosses with the SCN mtDNA; however, no pairwise differences were observed between generations (i.e. P₀, F₁, F₂, etc.) regardless of mtDNA source population. Furthermore, the variance in mtDNA copy number of the F₂ generation was far greater than any of the other generations in all crosses. Data grouped by each mtDNA source show significant heterogeneity of variance across all generations; this effect is eliminated by the removal of the F₂ generation (Table 1). Although there is no strong effect of hybrid or backcross generation on mtDNA copy number, variances were significantly different and skewed to higher values in the F₂ generation (Fig. 1 and Table 1). The observed variation in mtDNA content in hybrids is consistent with previous work showing increased variance fitness in interpopulation F₂ hybrids of *T. californicus*, although the relationship between mtDNA content and hybrid breakdown in fitness was not explicitly addressed in this study.

mtRPOL genotype-specific variation of mtDNA copy number

All animals in the F₂ hybrid and maternal and paternal F₂ hybrid backcross generations were assayed for mtDNA copy number and genotyped for mtRPOL. mtRPOL genotypes were classified as ‘matched’ if homozygous for the mtRPOL allele derived from the same parental population as the mtDNA, as ‘mismatched’ if homozygous for the mtRPOL allele derived from a different parental population than the mtDNA or as ‘heterozygote’

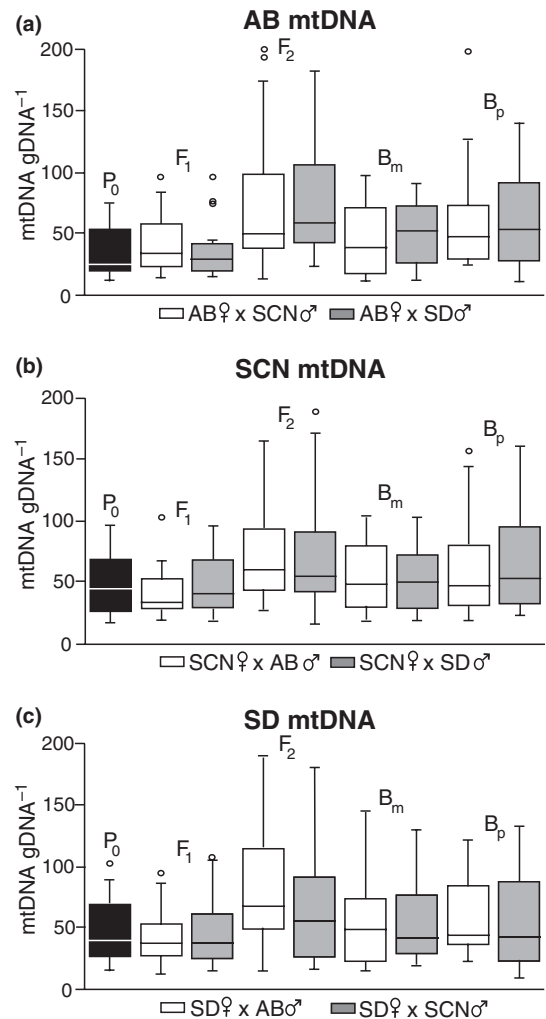


Fig. 1 Box-and-whisker plots of mitochondrial DNA copy number in P₀ (parental), F₁ and F₂ (interpopulation hybrids) and B_m (maternal) and B_p (paternal) backcross generations.

Table 1 ANOVA and Levene’s homogeneity of variance test results for mitochondrial DNA (mtDNA) copy number data grouped by mtDNA genotype.

mtDNA	d.f.	F	P	L	P
All data					
AB	179	2.846	0.005	3.521	0.001
SCN	179	1.696	0.092	3.521	0.046
SD	179	1.525	0.04	2.075	0.038
Excluding F ₂ generation					
AB	139	0.53	0.785	1.189	0.316
SCN	139	1.576	0.159	1.307	0.258
SD	139	0.797	0.574	1.152	0.336

d.f., degrees of freedom; ms, mean square error; F, F-statistic; L, Levene’s statistic. Significant results in all cases are eliminated by removal of F₂ animals from the analysis. Note that no ANOVA *post hoc* pairwise comparisons were significant in this case ($\alpha = 0.05$).

if heterozygous for mtRPOL. Within each mtRPOL genotype and cross, no significant differences were found for mtDNA copy number between the cross types (data not shown). Therefore, within each mtRPOL genotype, data from the F_2 , maternal and paternal backcross generations were pooled within each cross and all data presented in Fig. 2 and Table 2 refer only to mtRPOL genotype, independent of hybrid or backcross genetic background (parental lines are excluded).

In hybrids with either the AB or SCN mtDNA genotype, mtDNA copy number was not significantly different in matched and heterozygous animals, although both

genotypes had significantly lower mtDNA copy number than did the mismatched mtRPOL–mtDNA genotype (Table 2). This stands in contrast to the results from hybrids bearing the SD mtDNA genotype in two important ways. First, animals with the matched SD mtRPOL–mtDNA genotype had significantly higher mtDNA copy number than either the matched or heterozygote genotypes, an inverse pattern from that observed in animals with either AB or SCN mtDNA. Second, animals heterozygous for mtRPOL had significantly higher mtDNA copy number than mismatched mtRPOL–mtDNA genotype animals, whereas these two genotypes were more similar in hybrids with either AB or SCN mtDNA. Although the response of mtDNA copy number to mtRPOL genotypes is not consistent in all crosses studied, mtRPOL genotype clearly impacts mtDNA copy number in each case.

mtDNA copy number and mtRPOL genotype impact mitochondrial transcription

Mitochondrial transcription is primarily mediated by mtRPOL and the mitochondrial promoter (mtDNA). For each of the six interpopulation hybrid crosses in this study, we compared ratios of ATP6 transcripts with ATP6 gene dosage (mtDNA copy number) in standard culture condition control vs. hypo-osmotic stress conditions. Data on transcript levels were collected as part of a separate study evaluating transcription in hybrids independent of mtDNA copy number (Ellison & Burton, 2008b). Data are presented in Fig. 3 and Table 3 grouped by mtDNA source population and mtRPOL genotypes.

Regardless of mtDNA source, transcript levels under hypo-osmotic stress were significantly higher than in control conditions for each of the parental genotypes. In hybrids with either the AB or SCN mtDNA genotype, the same was true in hybrids with the matched

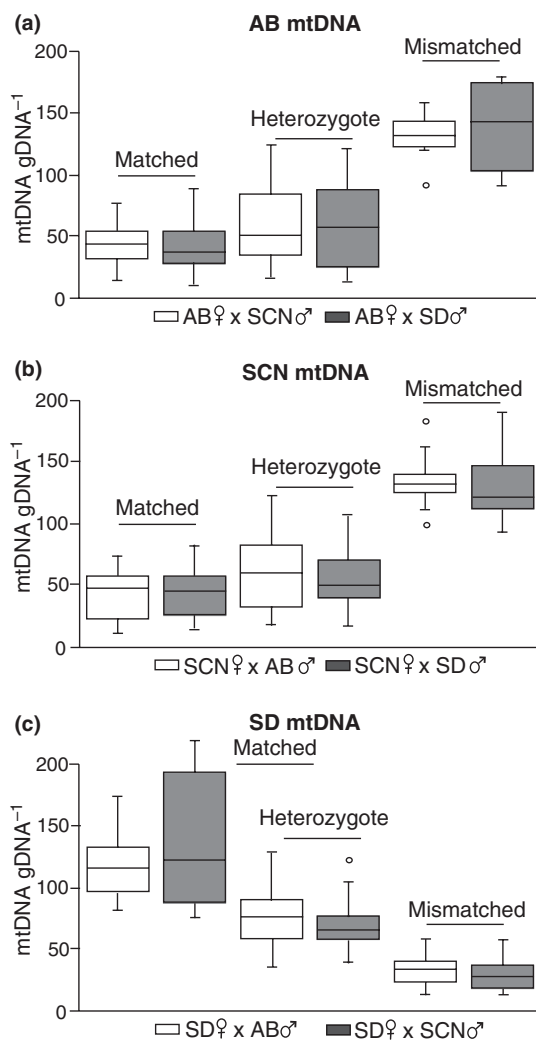


Fig. 2 Mitochondrial RNA polymerase (mtRPOL) genotype influences mitochondrial DNA (mtDNA) copy number in interpopulation hybrids. Box-and-whisker plots with data grouped by mtDNA genotype and cross. Matched, mtRPOL and mtDNA derived from the same parental population; heterozygote, heterozygous mtRPOL; mismatched, mtRPOL and mtDNA derived from different parental populations.

Table 2 Mitochondrial RNA polymerase (mtRPOL) genotype impacts mitochondrial DNA (mtDNA) copy number in interpopulation hybrids. Data are grouped by mtDNA genotype and cross. Pairwise comparisons of mtDNA copy number across mtRPOL genotype within each cross are presented (Tamhane's T2 test; $\alpha = 0.05$). All crosses listed as (female parent) \times (male parent).

	Heterozygote	Match	Mismatch
AB \times SD (above diagonal) and AB \times SCN (below)			
Heterozygote	–	0.064	<0.001
Match	0.147	–	<0.001
Mismatch	0.005	0.003	–
SCN \times SD (above diagonal) and SCN \times AB (below)			
Heterozygote	–	0.071	<0.001
Match	0.27	–	<0.001
Mismatch	<0.001	<0.001	–
SD \times AB (above diagonal) and SD \times SCN (below)			
Heterozygote	–	0.004	<0.001
Match	0.003	–	0.001
Mismatch	<0.001	<0.001	–

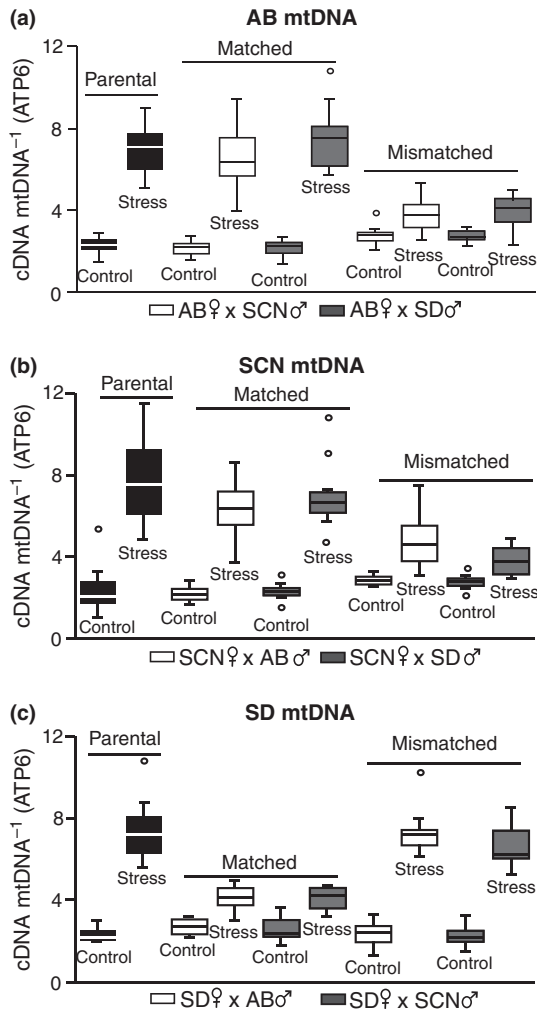


Fig. 3 Interaction of mitochondrial DNA (mtDNA) copy number and mitochondrial RNA polymerase (mtRPOL) genotype influences transcriptional regulatory stress response of mitochondrial genes in interpopulation hybrids. Expression of the mitochondrial-encoded ATP6 gene in parental, matched and mismatched mtRPOL hybrids normalized to mtDNA copy number is shown for control and hypo-osmotic stress conditions in box-and-whisker plots. Certain mtRPOL–mtDNA genotypic combinations have reduced hypo-osmotic stress response.

mtRPOL–mtDNA genotype. The difference between control and stress treatments was only marginally significant, if at all, for hybrids with mismatched genotypes (Table 3). Hybrids bearing the SD mtDNA genotype again showed an opposite pattern, in which transcript levels were significantly higher under hypo-osmotic stress only in hybrids with mismatched mtRPOL–mtDNA; the effect was virtually absent in hybrids with the matched genotype (Table 3). Two conflicting patterns are apparent in this data; however, it is clear that hybrids with certain mtRPOL–mtDNA genotypic combinations (mismatched for AB and SCN mtDNA; matched for SD mtDNA) have a greatly decreased ability to increase the transcript to gene ratio when under hypo-osmotic stress.

Discussion

Variation in mtDNA copy number

Mean mtDNA copy number does not change radically as a result of hybridization; the variance around this mean, however, is significantly higher in F₂ hybrids than in parents, F₁ hybrids or F₂ backcross animals. This variance can be partitioned by mtRPOL genotype and large differences in mtDNA copy number are observed in different genotypic combinations. Although it is certainly possible that another gene, tightly linked to the mtRPOL locus, could be responsible for the observed patterns, the known role of mtRPOL in mtDNA transcription and replication in animals makes this a strong candidate gene. Notably, Flowers (2005) found that the mitochondrial transcription factor mtTFB1 is tightly linked to mtRPOL locus in *T. californicus* and would remain in linkage disequilibrium over the 2–3 generations of crosses involved in the current experiments; as both loci show population-specific nucleotide variation, we are not at this point able to definitively ascribe the observed phenotypes to mtRPOL alone.

In hybrids with either the AB or SCN mtDNA genotype, mismatched mtRPOL–mtDNA genotype combinations are associated with a higher mtDNA copy number, whereas mtDNA copy numbers in matched and heterozygote genotypes are both lower and approximately equivalent. Thus, any effect of mtRPOL on mtDNA copy

Table 3 Mitochondrial RNA polymerase (mtRPOL) genotype impacts ability to up-regulate mitochondrial genes relative to mitochondrial DNA (mtDNA) copy number in response to hypo-osmotic stress. ANOVA significance of control vs. hypo-osmotic stress treatment for each genotype, within each cross, is shown. Degrees of freedom and *F*-statistic are listed in parentheses (d.f., *F*).

Genotype	Population	<i>P</i>	Population	<i>P</i>	Population	<i>P</i>
Parent	AB	<0.001 (23, 26.0)	SCN	<0.001 (23, 21.3)	SD	<0.001 (23, 32.9)
Matched hybrid	AB × SCN	<0.001 (23, 29.1)	SCN × AB	<0.001 (23, 24.4)	SD × AB	0.044 (18, 4.7)
	AB × SD	<0.001 (20, 36.8)	SCN × SD	<0.001 (23, 19.0)	SD × SCN	0.037 (23, 8.8)
Mismatched hybrid	AB × SCN	0.021 (23, 6.1)	SCN × AB	0.043 (23, 4.6)	SD × AB	<0.001 (18, 19.7)
	AB × SD	0.028 (19, 5.7)	SCN × SD	0.054 (23, 4.1)	SD × SCN	<0.001 (23, 27.2)

number appears to require a homozygous mismatched genotype in these cases. This further suggests that a single nuclear gene copy is sufficient to re-establish the association between mtDNA copy number phenotype and mtRPOL genotype. This dominant effect is consistent with the results of Ellison & Burton (2008a). However, crosses with the SD mtRPOL showed a stepped pattern in which mtDNA copy number decreased sequentially from matched mtRPOL–mtDNA genotype individuals, to heterozygotes and finally to mismatched individuals. This is a more complex pattern than that observed in hybrids with AB or SCN mtDNA and suggests an incomplete dominance effect of mtRPOL, or another tightly linked locus, on mtDNA copy number. Although hybrids with SD mtDNA present a contrasting pattern to that of hybrids with either AB or SCN mtDNA, the results are consistent with previous work showing that the hybrid mtRPOL genotypes with elevated mtDNA copy number in this study are the same as those with failed transcriptional stress response under hypo-osmotic stress (Ellison & Burton, 2008b). Wild-type mtDNA–mtRPOL genotypes having deleterious effects in a hybrid context (e.g. the matched SD mtDNA–mtRPOL genotype here) suggest that a more complex epistatic network of loci contribute to the regulation of mtDNA copy number in *T. californicus* interpopulation hybrids. These likely include deleterious nuclear–nuclear interactions (Willet, 2006; see also Edmands *et al.*, 2009). A similar mechanistic argument was presented by Ellison & Burton (2008b) for a parallel pattern in the mitochondrial transcriptional regulatory network. Nevertheless, the data here indicate that the interaction between mtRPOL and mtDNA has a measurable impact on the regulation of mtDNA copy number.

It is unclear what direct influence, if any, mtRPOL genotype has on changes in mtDNA copy number in *T. californicus* interpopulation hybrids. Mechanisms controlling mtDNA replication and copy number are relatively poorly understood (Moraes, 2001; Scheffler, 2008). mtDNA replication is primed with an RNA primer generated by mtRPOL and proceeds with the mtDNA polymerase. A large number of additional nuclear-encoded accessory proteins are also involved in mtDNA maintenance, recombination and repair, but are not essential to replication (Moraes, 2001). At least two of these, TFAM and twinkle helicase, are known to directly regulate mtDNA copy number (Ekstrand *et al.*, 2004; Tynismaa *et al.*, 2004) and specific mutations in TFAM are known to significantly affect mtDNA copy number (Suissa *et al.*, 2009). Additionally, a set of termination factors that impact transcription and regulation of mtDNA, the MTERF family of proteins, are likely key components of the mtDNA regulatory system (Roberti *et al.*, 2009). Finally, concentrations of reactive oxygen species in the mitochondrion may play an important role in determining rates of mtDNA replication (Hori *et al.*, 2009). This study does not have the resolution to distinguish between these diverse regulatory components

and, in fact, many are unknown in the *Tigriopus* system. This does not, however, diminish the likely role of mtRPOL in disrupting hybrid mtDNA regulation. Regardless of the mechanism, it is clear that changes in mtDNA copy number are associated with a number of human disease phenotypes and are also essential for normal physiological processes (Moraes, 2001; Rantanen *et al.*, 2001). However, human pathologies associated with mtDNA copy number typically involve depletion of mtDNA (Elpeleg *et al.*, 2002; Jeng *et al.*, 2007). Although increased mtDNA copy number in *T. californicus* interpopulation hybrids may affect fitness in subtle, but possibly consequential, ways, it seems unlikely that this decrease contributes directly to decreased hybrid fitness in a manner analogous to human mtDNA depletion syndromes. Instead, increased mtDNA copy number in certain low-fitness hybrids seems to reflect a dysfunction of the control mechanisms that normally regulate mtDNA copy number.

Mitochondrial transcriptional compensation

Both parental *T. californicus* and interpopulation hybrids have increased transcript levels of OXPHOS loci, including polypeptides encoded in the mitochondrial genome, when under hypo-osmotic stress. Ellison & Burton (2008b) showed that the increases in mtDNA transcripts were strongly impacted by mtRPOL genotype. Here, we have shown that, depending on the hybrid mtRPOL genotype, mtDNA copy number varies significantly and, consequently, the cellular availability of mtRPOL promoter-binding sites also varies. Initiation of mitochondrial transcription is critically dependent on the interactions between mtRPOL and the mitochondrial promoter region (Gaspari *et al.*, 2004; Matsunaga & Jaehning, 2004); although gene dosage is known to have a profound effect on gene expression (Birchler *et al.*, 2005; Jeng *et al.*, 2007), it is unclear what impact the observed change in mitochondrial gene dosage (mtDNA copy number) may have on mitochondrial gene expression.

Transcript levels in control and hypo-osmotic stress treatment animals were normalized to mtDNA copy number to determine whether gene dosage contributes to the dysfunction of the mitochondrial regulatory system in interpopulation hybrids. The same hybrid mtRPOL genotypes associated with increased mtDNA content were found to have decreased response to hypo-osmotic stress. Although these results are similar to the mtRPOL genotype-dependent stress response in terms of absolute transcript number (i.e. not corrected for mtDNA content; Ellison & Burton, 2008b), the magnitude of the effect is amplified by the inclusion of mtDNA copy number data. Deleterious hybrid mtRPOL genotypes, mismatched mtRPOL in hybrids with AB or SCN mtDNA and matched mtRPOL in hybrids with SD mtDNA, have a transcriptional stress response that is

only marginally significant when normalized to mtDNA copy number; other hybrid genotypes and parental lineages have a clearly significant response. This suggests that hybrids bearing these context-dependent deleterious mtRPOL genotypes may be near maximal mitochondrial transcriptional capacity even under control conditions.

Constant ratios of mtDNA to total cellular DNA under several growth conditions in human cell lines indicate that these cells likely carry an excess of mtDNA in standard conditions and draw on this excess when faced with physiological stress (Shay *et al.*, 1990). However, certain environmental conditions (e.g. calorie restriction) may promote mitochondrial biogenesis and up-regulate genes targeted to the mitochondria (Hock & Kralli, 2009) and, although mtDNA copy number often correlates with organelle number within specific tissues, this relationship does not always hold (e.g. Sogl *et al.*, 2000). Overall mtDNA copy number in *T. californicus* individuals, regardless of mtRPOL genotype, is several fold lower than in many mammalian systems (e.g. Rantanen *et al.*, 2001; Elpeleg *et al.*, 2002; Miller *et al.*, 2003; Ekstrand *et al.*, 2004; Jeng *et al.*, 2007). The significance of this is unclear, although mtDNA copy number may be a plastic character. Willett (2008) suggests that initial rearing conditions can dramatically alter patterns of selection observed in *T. californicus* hybrids. Still, the relative reduction of mtDNA copy number in *T. californicus* does not appear to have any of the strongly deleterious effects seen in human mtDNA depletion syndromes, as parental animals maintain the lowest mtDNA copy numbers observed in this study. Regulation of mtDNA copy number involves many components (Moraes, 2001; Holt *et al.*, 2007), but replication is known to require initiation by mtRPOL (Falkenberg *et al.*, 2007). Hence, reduced efficiency of the mtRPOL–mtDNA promoter interaction may contribute to changes in mtDNA copy number in *T. californicus* interpopulation hybrids. Increased mtDNA copy number in hybrids is associated only with those mtRPOL genotypes that are selected against in hybrids and that have reduced capacity for hypo-osmotic stress response (Ellison & Burton, 2008b). Although reduced mitochondrial transcriptional function may be partially buffered by increased mtDNA copy number under control conditions, it appears to be insufficient when faced with physiological stress. However, given that cells typically carry an excess of mtDNA and that mtDNA copy number appears to be an extremely plastic character in *T. californicus*, transcriptional dosage compensation cannot fully explain the broad variation in mtRPOL genotype-dependent mtDNA copy number observed in this study.

Regulatory cytonuclear conflict mediation

Interactions between the cell, or nucleus, and mitochondria must necessarily be conceptualized both in

terms of synergistic and antagonistic effects on fitness of the organelle, the cell and the organism as a whole (Blackstone, 1995). Mitochondria are often thought of as strictly providing an energetic service to the cell (i.e. a synergistic cytonuclear interaction), but the endosymbiotic origins of mitochondria suggest that conflicting levels of selection may be acting antagonistically on the mitochondrial and nuclear genomes (Cosmides & Tooby, 1981; Birky, 1995; Blackstone & Green, 1999; Rand, 2001; Burt & Trivers, 2006). Although intracellular conflict is minimized by uniparental inheritance of mtDNA and predominantly nuclear control of mitochondrial function (Birky, 1995), a vestige of ancient selfish nuclear–mitochondrial conflict can be seen in mitochondrial control of apoptosis (Kroemer, 1997; Blackstone & Green, 1999; Brown, 2005). Nuclear conflict modifiers are likely to have evolved to ameliorate this conflict between inherently different nuclear and mitochondrial interests to ensure for the survival of the cell, and by extension the organism (Michod & Roze, 2001), but no mechanistic examples have been proposed to date.

Nuclear–mitochondrial conflict can be framed more broadly as a selfish conflict between cellular energetic demands and mitochondrial proliferation. Rates of ATP synthesis are critically dependent on the diffusion of ions and substrates and, as such, on the number and shape of mitochondrial inner membrane cristae junctions and the density of OXPHOS enzyme complexes, parameters largely independent of mtDNA abundance (Frey & Mannella, 2000; Paumard *et al.*, 2002). Regulation of mtDNA abundance, via replication machinery, is complex and involves a dedicated enzyme complex. Although transcription of mtDNA is achieved through mtRPOL, the same enzyme may exhibit secondary influence on replication as initiation of replication requires a mtRPOL-generated RNA primer (Falkenberg *et al.*, 2007). It has been suggested that mutations affecting mtDNA transcription may also affect mtDNA replication in this manner (Clayton, 2000; but see Suissa *et al.*, 2009). Changes in the strength of interaction between mtRPOL and either the mtDNA promoter region or the mtDNA origin of replication owing to interpopulation hybridization may thereby bias mitochondrial regulation in favour of transcription of OXPHOS loci or replication of mtDNA, respectively. In this way, the nuclear–mitochondrial détente normally established in cells may be disrupted in hybrids, initiating a cascade of effects, such as those observed in *T. californicus*, disrupting mitochondrial regulation (Ellison & Burton, 2008b), mitochondrial function (Rawson & Burton, 2002; Ellison & Burton, 2006; Harrison & Burton, 2006) and fitness (Burton, 1990; Edmands, 1999).

As isolated populations or species accumulate genetic divergence through time, hybrids between these isolated groups may be expected to show some degree of hybrid inviability (Harrison, 1990; Coyne & Orr, 2004);

often, this effect can be attributed to a cytoplasmic effect (e.g. Cruzan & Arnold, 1999; Abe *et al.*, 2005; Fishman & Willis, 2006; Ellison & Burton, 2008a; Lee *et al.*, 2008; Niehuis *et al.*, 2008; Arntzen *et al.*, 2009) and occasionally more specifically to antagonistic cytonuclear interactions (e.g. Rand *et al.*, 2001, 2006; Dowling *et al.*, 2007). Synergistic cytonuclear interactions between mitochondrial and nuclear genomes are essential for normal function of the mitochondrial OXPHOS system, the cell and the organism as a whole and disruption of these interactions in hybrids may contribute to larger-scale evolutionary processes (Burton *et al.*, 2006; Gershoni *et al.*, 2009). Here, we show that hybrid genotypic combinations of mtDNA and mtRPOL that have minimal transcriptional responsiveness to environmental stress also have elevated levels of mtDNA. mtRPOL may act as the tipping point, balancing cellular energetic needs (via mtDNA transcription) with mitochondrial replication. By disrupting coevolved mtRPOL, mtDNA promoter and mtDNA origin of replication combinations that exist at the population level, hybridization may then effectively impair nuclear-mitochondrial interactions, with deleterious consequences both for the cell and fitness of the hybrid individual. Although the precise molecular mechanisms of these interactions and their downstream effects on the organism remain unknown, mtRPOL clearly affects the distribution of mitochondrial regulatory functions in low-fitness interpopulation hybrids of *T. californicus*. This may represent a key mechanism contributing both to hybrid breakdown and, more broadly, to the maintenance of the cellular-mitochondrial symbiosis.

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