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Evolutionary genetics of hybrid breakdown in the marine copepod *Tigriopus californicus*

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Marine Biology

by

Christopher Kim Ellison

Committee in charge:

Professor Ronald S. Burton, Chair
Professor Lin Chao
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2008

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Chair

University of California, San Diego

2008

DEDICATION

To my parents, for unwavering support;

To my sister, for keeping me excited;

To Cécile, for keeping me sane

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

My first meeting with Ron Burton was, incongruously enough, on the morning of September 11, 2001, when I had arranged a meeting to discuss the prospects of entering the graduate program at Scripps Institution of Oceanography. Despite the forced awkwardness of meeting at such a time and, in retrospect, my utter cluelessness with regard to science, Ron somehow saw fit to invite me back to give graduate school a go. I am forever indebted to Ron for giving me the opportunity to work with him at Scripps Institution of Oceanography and also for the remarkable patience, incomparable guidance, and utterly inexplicable leeway that he has granted me.

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ABSTRACT OF THE DISSERTATION

Evolutionary genetics of hybrid breakdown in the
tidepool copepod *Tigriopus californicus*

by

Christopher Kim Ellison

Doctor of Philosophy in Marine Biology

University of California, San Diego, 2008

Professor Ronald S. Burton, Chair

Populations of the supra-littoral marine copepod *Tigriopus californicus* are known to be highly divergent and to exhibit a pattern of hybrid breakdown when crossed under laboratory conditions. This dissertation examines the genetic mechanisms involved in hybrid breakdown in *T. californicus*, particularly those involving integration of the nuclear and mitochondrial genomes. In Chapter I, I summarize some of the relevant literature concerning the importance of hybrid breakdown to evolutionary biology, the integration of nuclear and cytoplasmic components in mitochondria, and the

use of *T. californicus* as a model system. Chapter II reports on the results of an experiment mapping interpopulation hybrid breakdown in *T. californicus* to the mitochondrial genome. Using a simple backcrossing scheme, this work determines that virtually the entire effect of hybrid breakdown is explained by mitochondrial genotype in hybrids. While an extension of the work examining mitochondrial biochemical capacity yielded a similar result, the results further indicated that the cause of hybrid breakdown in *T. californicus* likely involved more complex incompatibilities. Chapter III demonstrates that cytonuclear hybrid incompatibilities can manifest themselves in the biochemical performance of mitochondria. Activities of the primary enzyme complexes involved in cellular energy generation were measured in hybrids, as was the overall mitochondrial energy production capacity. Mitochondrial ATP production rate was reduced in hybrids, but only those enzyme complexes requiring the interaction of nuclear and mitochondrial gene products were similarly reduced. This study suggests that failure to integrate nuclear and mitochondrial gene products in hybrids may decrease their capacity to generate cellular energy. Chapter IV examines the impact of hybridization on the nuclear and mitochondrial transcriptional regulatory networks, specifically with regard to genes involved in cellular energy generation. Mitochondrial RNA polymerase genotype is found to have a profound impact on the transcriptional pattern of *T. californicus* interpopulation hybrids such that particular combinations of mitochondrial RNA polymerase and mitochondrial DNA have a diminished ability to upregulate mitochondrial genes under hypoosmotic stress. The data indicate that the mitochondrial regulatory network may be maintained by compensatory mechanisms in some populations.

CHAPTER I

Introduction

The genetic basis of hybrid breakdown

As a consequence of the combined action of selection, mutation, and random genetic drift, allopatric populations are expected to gradually diverge over time. If such divergent populations subsequently hybridize, the hybrid offspring often suffer some degree of infertility or inviability. Importantly, this reduction of hybrid fitness may contribute to the process of speciation by preventing or reducing introgression between parental populations or species. One major recent focus of evolutionary biology research has been the elucidation of mechanisms driving hybrid breakdown, sterility, and inviability (Coyne and Orr 2004). Many studies have shown the importance of ecology (e.g. Rundle and Schluter 1998; Nosil et al 2002), behavior (e.g. Filchak et al 2000), and development (e.g. Peichel et al 2001; Colosimo et al 2005) in this process; here, however, I will focus only on the genetic basis of hybrid breakdown.

In the absence of ecological, behavioral, or development interactions, intrinsically low hybrid fitness is widely attributed to deleterious epistatic interactions between divergent parental genes or genomes in hybrids (Noor and Feder 2006; Bolnick et al 2008). The most common model for understanding the evolution of such incompatibilities is the Dobzhansky-Muller model (Dobzhansky 1936; Muller 1942; further developed by Orr 1993, 1995; Turelli and Orr 1995, 2000; Orr and Turelli 2001). Under this model, isolated populations independently fix divergent alleles at two or more loci. Though the mutations generating these novel alleles are not strongly deleterious, and may even be advantageous, in the respective genomic context in which they arise, in combination they are effectively untested by selection. As such, generation of novel genetic combinations in hybrids may create deleterious epistatic effects that ultimately

impair hybrid function. Several incompatibility loci thought to fall into this category have been identified (Ting et al 1998; Barbash et al 2003; Presgraves 2002; Presgraves et al 2003), though the pairwise interactions involved are largely uncharacterized (however, see Schartl et al 1999 and Brideau et al 2006 for significant exceptions).

Evolutionary biologists have long struggled to explain how reproductively isolated species can arise without the evolution of unfit intermediates. One of the primary attractions of the Dobzhansky-Muller model is that it provides a theoretical construct to explain the origin of deleterious hybrid traits that does not require either parental population to pass through a low fitness intermediate while accumulating divergent alleles (Coyne and Orr 2004). While the intuitiveness of the Dobzhansky-Muller model has led to its widespread acceptance, it is not the only model describing the evolution of hybrid incompatibility. An alternative model, involving compensatory evolution, was proposed by Kimura (1985). Under this model, deleterious mutations are balanced, or compensated for, within lineages by secondary mutations that yield the overall epistatic network neutral or nearly neutral. Hybridization then separates mutations from their compensatory epistatic network, thereby exposing their deleterious effects to selective pressure. The first mutation, which must be deleterious in this scenario, is unlikely to fix in large populations with high efficiency of natural selection and, thus, the conditions under which secondary, compensatory mutations are favored are generally considered to be relatively restricted. Some more recent models, however, show that under certain demographic scenarios, compensatory evolution may be much more likely than initially suspected (e.g. Carter and Wagner 2002; Weinreich and Chao 2005).

Interactions between nuclear and cytoplasmic genes (i.e. mitochondrial or chloroplast genes) constitute a minority of the total epistatic interactions present in any cell. However, certain characteristics of cytoplasmic genomes suggest *a priori* that nuclear-cytoplasmic epistasis may be more prone to dysfunction in hybrids. First, cytoplasmic genes are generally inherited solely through the maternal lineage, as opposed to the Mendelian pattern of inheritance in nuclear genes (Avise 1991). Consequently, a much larger fraction of F_2 hybrid progeny are expected to show the effects of a deleterious nuclear-cytoplasmic interaction than of an equivalent nuclear-nuclear genic interaction. Second, mitochondrial genes have a greatly elevated mutation rate relative to nuclear genes in many organisms (Brown et al 1979), sometimes up to 100-fold higher in the same species (Johnson et al 2003). As a result, isolated lineages may accumulate divergence in mitochondrial genes much faster than in nuclear genes. These mutations may then constitute the basis of genetic incompatibility in the event of hybridization between populations. While genetic analyses of hybrid incompatibility have, to a large degree, focused only on epistatic interactions between nuclear genes, an increasing number of studies suggest that nuclear-mitochondrial incompatibilities may play a central role in hybrid breakdown (Breeuwer and Werren 1995; Edmands and Burton 1999; Sackton et al 2003; Perrot-Minnot et al 2004; Zeyl et al 2005).

In this dissertation, I examine how hybridization of highly divergent, but reproductively compatible, populations affects nuclear-mitochondrial epistasis. This work suggests that interactions between nuclear and cytoplasmic genes may be an important, and often unrecognized, component of intrinsic postzygotic isolation.

Mitochondrial function in the cell

Mitochondria are often summarily described, both in textbooks and in primary literature, as the "powerhouses of the cell". This is an accurate description that also massively understates the role of mitochondria in normal cellular function. In fact, mitochondrial functions are diverse, including maintenance of cytosolic ion concentrations and cellular redox potential, initiation of apoptosis, and production both reactive oxygen species and ATP, and have broadly pleiotropic effects both on cells and organisms as a whole (Scheffler 2008). The endosymbiotic theory, postulating that mitochondria originated from aerobic, free-living α-proteobacteria, is now a widely accepted explanation of the cellular origin of mitochondria (Gray 1992; Boussau et al 2004). Over the course of the evolution of this cellular-mitochondrial symbiosis, many formerly mitochondrial genes have been translocated to the nuclear genome and many more nuclear genes have acquired derived functions and targeting signals specific to the mitochondrion (Rand et al 2004). Though most metazoan mitochondrial genomes contain a set of thirteen protein coding genes, the degree of mitochondrial genome reduction varies widely across taxa, with the protozoan *Reclinomas americana* having ninety-seven protein coding genes compared to a mere three protein coding genes in the malarial parasite *Plasmodium falciparum* (Berg and Kurland 2000).

The number of different nuclear-encoded polypeptides present in mitochondria has been estimated to exceed one thousand (Schatz 1995). This stands in stark contrast to the mere thirteen polypeptides typically encoded in metazoan mitochondrial genomes. Nevertheless, the integration of nuclear and mitochondrial encoded genes in the mitochondrion, with radically different inheritance, selection pressure, regulatory

mechanisms, and cellular localization, remains essential for mitochondrial function. All thirteen polypeptides encoded by the mitochondrial genome comprise integral components of the mitochondrial oxidative phosphorylation (OXPHOS) pathway that directly interact with approximately seventy nuclear encoded components in multimeric enzyme complexes (Grossman et al 2004; Scheffler 2008). The efficacy of these interactions, both between nuclear and mitochondrial encoded genes and between genes within either genome, influences the function and energy generation capacity of the mitochondrion and mutations resulting in deleterious interactions between genomes are likely to be strongly selected against.

Divergent taxa may be predisposed to intrinsic incompatibilities between nuclear and mitochondrial genes (Dey et al 2000). Coevolution of these genes within lineages may be extensive and has recently been explored using both primate and murid xenomitochondrial cell lines. For example, human cell lines harboring orangutan-derived mitochondria were found to be wholly incapable of oxidative phosphorylation, whereas the same cell lines with either chimpanzee or gorilla-derived mitochondria had functional OXPHOS pathways with reduced activities of complexes I and IV (Kenyon and Moraes 1997; Barrientos et al 2000). A similar study, using mouse cell lines, found that a functional OXPHOS system was only present in lines containing conspecific mitochondria, while mouse cell lines with either rat or shrew-derived mitochondria were strikingly deficient in the activities of complexes III and IV (McKenzie et al 2003). In sum, these studies suggest that nuclear and mitochondrial genes in the OXPHOS pathway are coadapted within species and are sensitive to a disruption of this coadaptation to a remarkable degree.

Interactions between the nucleus and mitochondrion are by no means limited to the protein-protein interactions described above and also include protein-nucleic acid interactions inherent in regulatory processes. Both replication and transcription of mtDNA are closely related to mitochondrial function and biogenesis (Bonawitz et al 2006) and, in contrast to nuclear genomic organization, the essential regulatory features (i.e. promoter regions) are all contained within a single portion of the mitochondrial genome called the control region or displacement loop (Asin-Cayuela and Gustafsson 2007). Transcription is accomplished by a dedicated, single subunit RNA polymerase acting in concert with up to two transcription factors (Tiranti et al 1997). Unusually, however, the core polymerase itself is responsible for promoter binding affinity and specificity and the transcription factors do not appear to directly play a role in promoter recognition. The interaction of the mitochondrial RNA polymerase and mitochondrial promoter is highly lineage specific, such that transcription can be initiated *in vitro* only with mitochondrial RNA polymerase and mitochondrial promoters from the same species (Gaspari et al 2004; Matsunaga and Jaehning 2004). Thus, coadaptation within the mitochondrial regulatory network appears to evolve in isolated lineages.

Mitochondrial function can have wide-ranging effects on cellular physiology that are a direct consequence of disrupted mitochondrial biochemistry. Examples include the collapse of mitochondrial membrane potential often associated with a signaling cascade leading to apoptosis (Kroemer et al 1998), specific mtDNA mutations that are linked to a number of human neurodegenerative disorders (Wallace 1992), and overproduction of reactive oxygen species contributing to aging and physiological decline (Harman 1957; Beckman and Ames 1998). Reactive oxygen species generated by mitochondrial

processes are generally the result of sub-optimal function of the OXPHOS pathway, with the majority of reactive oxygen species being generated as byproducts enzyme complexes I and III (Balaban et al 2005). In this way, failure of one component of mitochondrial function can trigger pathways that affect the organism as a whole.

In this dissertation, I discuss coadaptation of nuclear and mitochondrial genes within isolated lineages. Disrupting these coadapted gene complexes via hybridization, I evaluate some of the biochemical and regulatory consequences of hybridization on mitochondrial function as well as organismal fitness.

Tigriopus californicus as a model system

Tigriopus californicus is a harpacticoid copepod inhabiting supra-littoral tidepools on the west coast of North America with a range stretching approximately from central Baja California, Mexico, to southern Alaska in the United States. Copepods in the genus *Tigriopus* are found globally in the upper intertidal of rocky coasts (Boxhall and Halsey 2004) and are known to have extraordinary tolerance of salinity and temperature extremes (Damgaard et al 1994; Davenport et al 1997; Edmands and Deimler 2004). The physiology of osmoregulation in *T. californicus* is well studied (Burton and Feldman 1983; Burton 1986, 1991a, b; Goolish and Burton 1988, 1989) and the *Tigriopus* genus is increasingly used as a model marine system both for toxicology studies (e.g Bengtsson 1978; O'Brien et al 1988; Kwok and Leung 2005; Lee et al 2007) and as an experimental vector for pathogenic bacterial strains (e.g. Mueller et al 2007).

Populations of *T. californicus* are highly differentiated. At one allozyme locus, phosphoglucoisomerase, two populations separated by only 500 meters of sandy beach have maintained private alleles over nearly three decades of study, indicating zero gene flow even over very short spatial scales (Burton and Feldman 1981; Burton 1997; Flowers 2005). Nucleotide sequencing efforts have further demonstrated a remarkable degree of sequence divergence, particularly of mitochondrial loci, which can exceed 18% uncorrected interpopulation divergence at cytochrome oxidase I (Burton and Lee 1994; Burton 1998; Edmands 2001; Burton et al 2007). Rand et al (2004) suggest that small effective population sizes of *T. californicus* result in weaker natural selection and, consequently, increased fixation rate and highly divergent populations. However, efforts to determine the effective population size of two northern California populations were

largely inconclusive (Flowers 2005) and, in fact, populations found in the northern end of the range have far less interpopulation sequence divergence than more southerly populations despite similar life histories, suggesting that effective population size may not completely explain the phenomenon (Edmands 2001). Regardless of the cause, it is clear that populations of *T. californicus* harbor an extreme degree of interpopulation mitochondrial sequence divergence.

T. californicus exhibit a mate guarding behavior common to the genus in which mature males clasp juvenile female copepodids until they are reproductively mature (Burton 1986; Lazzaretto et al 1994; Kelly and Snell 1998). Further, females mate only once, so males always clasp virgin females; this behavior allows easy manipulation of breeding in the laboratory. Clasped males and virgin females can be teased apart using a fine needle and allowed to re-pair under controlled conditions. Widely diverged populations of *T. californicus* can thus be freely crossed under laboratory conditions. Interestingly, these crosses frequently result in interpopulation hybrids with increased fitness in the first generation (heterosis) and reduced fitness in the second hybrid generation (hybrid breakdown) (Burton 1990a, b; Edmands 1999). This pattern of F_2 hybrid breakdown implies that interactions between multiple loci are involved in the reduced fitness of hybrids. First generation hybrids, which are heterozygous at all nuclear loci, likely benefit from the masking of mildly deleterious recessive alleles from both parental populations. Independent assortment and recombination may then yield second generation hybrids with particular allelic combinations that generate deleterious epistatic interactions and, consequently, negatively impact hybrid fitness.

One class of interactions in which this may be manifested is epistasis of nuclear and mitochondrial genomes. Integration of nuclear and mitochondrial genomes has long been suspected to be negatively affected by interpopulation hybridization in *T. californicus* (Burton et al 1999; Blier et al 2001). Studies of cytochrome *c* oxidase activity and viabilities of cytochrome *c* genotypes in hybrids showed strong evidence that nuclear-cytoplasmic coadaptation was disrupted via hybridization (Edmands and Burton 1999; Willett and Burton 2001, 2003). Following on these studies, detailed biochemical assays demonstrated that functional coadaptation of cytochrome *c* and cytochrome *c* oxidase within *T. californicus* populations could be mapped to a single amino acid substitution (Rawson and Burton 2002; Harrison and Burton 2006). A suite of sequence analyses were able to distinguish further evidence of nuclear-mitochondrial coadaptation within populations, particularly elevated substitution rates among nuclear loci known to interact with mitochondrial loci (Willett and Burton 2004; Rawson and Burton 2006). Willett (2006) found additional evidence of the dissolution of nucler-nuclear epistasis in interpopulation hybrids, suggesting that both inter- and intra-genomic coadaptation may play important roles in hybrid breakdown. Finally, Flowers and Burton (2006) investigated the action of nucleolar dominance in hybrids and introduced a regulatory element to the mechanics of interpopulation hybrid breakdown.

In this dissertation, I study mechanisms underlying interpopulation hybrid breakdown in *T. californicus*. The primary focus is failure of nuclear-mitochondrial integration in these hybrids and the subsequent fitness consequences of these failures.

Conclusion

Further elucidation and understanding of the mechanisms of hybrid breakdown are of central importance to the study of evolution and the genesis of species. In chapters II, III, and IV, I explore aspects of nuclear-mitochondrial coadaptation in populations of *T. californicus* and the breakdown of that coadaptation in interpopulation hybrids from genetic, biochemical, and regulatory perspectives. In the second chapter, I employ a classical genetics crossing scheme to map the origin of interpopulation hybrid breakdown to the mitochondrial genome. The third chapter describes a series of experiments to elucidate the effects of failed nuclear-mitochondrial epistasis on mitochondrial biochemistry and performance. The final chapter examines mitochondrial transcriptional regulatory dysfunction in interpopulation hybrids and suggests that coadaptation of loci within *T. californicus* populations may evolve through a compensatory process. Together, these studies contribute to a general understanding of nuclear-mitochondrial coadaptation and its role in hybrid breakdown and, ultimately, the evolution of species.

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CHAPTER II

Interpopulation hybrid breakdown maps to the mitochondrial genome

Abstract

Hybrid breakdown, or outbreeding depression, is the loss of fitness observed in crosses between genetically divergent populations. The role of maternally inherited mitochondrial genomes in hybrid breakdown has not been widely examined. Using laboratory crosses of the marine copepod *Tigriopus californicus*, I report that the low fitness of F_3 hybrids is completely restored in the offspring of maternal backcrosses, where parental mitochondrial and nuclear genomic combinations are reassembled. Paternal backcrosses, which result in mismatched mitochondrial and nuclear genomes, fail to restore hybrid fitness. These results suggest that fitness loss in *T. californicus* hybrids is completely attributable to nuclear-mitochondrial genomic interactions. Analyses of ATP synthetic capacity in isolated mitochondria from hybrid and backcross animals found that reduced ATP synthesis in hybrids was also largely restored in backcrosses, again with maternal backcrosses outperforming paternal backcrosses. The strong fitness consequences of nuclear-mitochondrial interactions have important, and often overlooked, implications for evolutionary and conservation biology.

Introduction

Evolution in isolated populations can result in genetic differentiation that negatively impacts the fitness of interpopulation hybrids. Although first generation hybrids are frequently characterized by hybrid vigor, later generations often suffer dramatically reduced fitness (Dobzhansky 1970; Armbruster et al 1997; Galloway and Fenster 1999; Hall and Willis 2005). This pattern of F_2 hybrid breakdown can be regarded as an early stage of isolation between species and is most commonly attributed to negative interactions between loci known as Dobzhansky-Muller incompatibilities (Dobzhansky 1936; Muller 1942; Coyne and Orr 2004). Although several genes involved in Dobzhansky-Muller incompatibilities have been identified via genetic mapping approaches (Ting et al 1998; Barbash et al 2003; Presgraves et al 2003; Brideau et al 2006), the specific interacting loci and physiological processes driving hybrid breakdown remain largely unexplained.

To date, most genetic analyses of hybrid incompatibility have focused on interactions between nuclear genes. However, an increasing number of studies have suggested that nuclear-mitochondrial gene interactions may be particularly susceptible to disruption by hybridization (Breeuwer and Werren 1995; Edmands and Burton 1999; Sackton et al 2003; Perrot-Minnot et al 2004; Zeyl et al 2005). In fact, the often rapid rate of mitochondrial DNA (mtDNA) evolution in animals combined with the essential function of nuclear-mitochondrial gene interactions in cellular energy metabolism may predispose this system to dysfunction in hybrids, where the mitochondrial genome interacts with nuclear genes derived from a different population. Rand and colleagues (2005) have suggested a model of nuclear-mitochondrial interaction in which the

accumulation of deleterious mutations in mtDNA within a population is tolerated due to compensatory mutations in interacting nuclear genes. Under this model, F_1 hybrids contain a full haploid complement of nuclear genes coadapted to the mtDNA and suffer no loss of fitness, while recombination yields F_2 and later hybrids lacking the full set of compensatory mutations and, consequently, these later generation hybrids exhibit reduced fitness.

Populations of the marine copepod *Tigriopus californicus* exhibit high levels of mtDNA sequence divergence but retain the ability to produce viable hybrid offspring (Burton 1986; Edmands 1999). Laboratory crosses between *T. californicus* populations show a consistent pattern of F_1 hybrid vigor and F_2 hybrid breakdown for many metrics of physiological performance and fitness (Burton 1986, 1990; Edmands 1999; Burton et al 2006). I hypothesize that the pervasive nature of hybrid breakdown in *T. californicus* may result from the dysfunction of some fundamental cellular process resulting in broad pleiotropic effects. As discussed above, the interaction between nuclear and mitochondrial genomes that underlies mitochondrial energy production (Burton et al 2006) is an intriguing candidate system for such dysfunction in hybrids. The mitochondrial genome of *T. californicus* has been sequenced (Burton et al 2007) and, like most animal mtDNA, encodes 13 polypeptides, all of which are subunits of the enzyme complexes comprising the mitochondrial electron transport system (ETS). ETS activity requires functional interactions between each of the mitochondrial-encoded components and multiple nuclear-encoded ETS subunits. Specific functional interactions between nuclear-encoded cytochrome *c* and cytochrome *c* oxidase (ETS Complex IV) have demonstrated that *T. californicus* populations harbor coadapted sets of nuclear and

mitochondrial loci and that mismatches in hybrids can lead to disruption of physiological processes and reduced fitness (Rawson and Burton 2002; Harrison and Burton 2006). At the organellar level, mitochondrial energy production is reduced in *T. californicus* interpopulation hybrids, as is the activity of those ETS enzyme complexes composed of both nuclear and mitochondrial subunits (Ellison and Burton 2006). Together, these studies suggest that mitochondrial energy production plays an important role in hybrid breakdown, though it has been difficult to assess the quantitative contribution of nuclear-mitochondrial genomic interactions to reduced hybrid fitness.

Here I report the first direct test of the hypothesis that disruption of nuclear-mitochondrial gene interactions can account for the reduced fitness of interpopulation hybrids. The experimental strategy is simple: hybrids with low fitness are backcrossed to both maternal and paternal parental lines. Because mtDNA is maternally inherited, reciprocal backcrosses can be used to generate hybrids that, in the absence of strong selection, have identical average nuclear gene composition, but different mtDNA. I hypothesize that wildtype fitness requires the mtDNA and, at minimum, a full haploid nuclear genome from the same population. This hypothesis is rejected if both reciprocal backcrosses, or neither, result in the recovery of wildtype fitness and gains support if fitness recovery is observed only in the maternal backcross. Using this approach, I can quantitatively assess the role of nuclear-mitochondrial interactions in interpopulation hybrid breakdown.

Materials and Methods

Tigriopus culture conditions

Tigriopus californicus populations were sampled at three locations: Santa Cruz, California, USA (SCN: 36°57'N, 123°03'W, collected April 2006), Abalone Cove, Palos Verdes Peninsula, California, USA (AB: 33°44'N, 118°22'W, collected May 2006), and San Diego, California, USA (SD: 32°45'N, 117°15'N 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 liter filtered seawater. Animals were transferred to fresh dishes with each generation.

Mature male *T. californicus* clasp virgin females with their antennae until the females are reproductively mature; each female mates only once. Virgin females were separated from clasped males using a fine needle, then mated with males from the required population. Six interpopulation crosses were undertaken comprising all pairwise comparisons of the three populations listed above and their reciprocal crosses. For generations beginning with F_1 hybrids, pairs were removed from culture and crossed with individuals in a replicate Petri dish containing the same cross and same generation. All crosses were sufficiently replicated to employ a non-inbreeding strategy through the F_3 generation. Backcrosses were performed by crossing virgin F_3 hybrid females to males from either the original maternal or paternal population.

Life history and mitochondrial measurements

For each generation in this study (parental controls, F_1 hybrids, F_2 hybrids, F_3 hybrids, maternal backcrosses, and paternal backcrosses), fecundity, survivorship, and metamorphosis data were collected. Clasped pairs were placed in individual Petri dishes and the male was removed from the dish once the pair separated. Fecundity was measured as the number of nauplii hatching from a female's first eggsac. These nauplii were transferred to a new dish and observed again after 14 days; both the number of surviving individuals and the number of copepodids (juveniles) were counted. Survivorship was calculated as the fraction of first clutch nauplii surviving to 14 days and metamorphosis fraction was calculated as the percentage of first clutch nauplii that had developed into copepodids after 14 days. All data were collected for ten replicate clutches for each cross at each generation. Animals were cultured under common garden conditions at 20°C.

ATP production by isolated mitochondria was determined for each of the clutches according to the protocol of Ellison and Burton (2006). Briefly, all adult individuals from a clutch were pooled 18 to 24 days after hatching. Intact, functional mitochondria were extracted from these pooled animals and used to measure rate of ATP production in a five-minute end point assay with exogenous ADP, pyruvate, and malate substrates (compared to a blank with no added substrate). Data were normalized to protein content in the mitochondrial preparation.

Statistical analysis

All statistical analyses were completed using the SPSS 11.0 statistics package. One-way ANOVA was used for each comparison with a post-hoc Bonferroni test using an alpha value of 0.05 for five comparisons within each of the six interpopulation hybrid classes for fecundity, survivorship, metamorphosis fraction, and ATP production rate data: maternal population backcross versus F_3 hybrid, paternal population backcross versus F_3 hybrid, maternal population backcross versus paternal population backcross, maternal population backcross versus maternal population, and paternal population backcross versus maternal population. Comparisons of backcrosses versus midparent values were calculated, but were not qualitatively different than the maternal population comparisons (Table 2.1) and are not reported. All measures were analyzed with the units described above. n = 10 for each category of data (i.e. 10 replicates of each measure for each cross in every generation). Equality of variance was tested using Levene's test of homogeneity of variances; no significant deviations were found (alpha = 0.05) Normal distribution of data was tested using a Kolmorogov-Smirnov test for goodness of fit with Gaussian parameters; no significant deviations from normality were found (alpha = 0.05).

Results

All six pairwise crosses were initiated among three natural populations of *T. californicus*. Fecundity, survivorship, and metamorphosis rate were recorded for each of these crosses at the F_1, F_2, and F_3 generations to determine fitness, then F_3 females were backcrossed to both maternal and paternal parental populations and fitness measures were repeated. In all six cases, fitnesses of F_1 offspring were equal to or greater than that of either parental lineage. In contrast, fitnesses declined significantly in the F_2 generation and showed no recovery in the F_3 generation (Figures 1.1, 1.2, and 1.3). *T. californicus* have 12 chromosomes and lack recombination in females (Ar-Rushdi 1963; Burton et al 1981), so the combined effect of independent assortment and recombination effectively ensured that none of the F_3 offspring would have a full haploid complement of alleles from either parental population. Hybrid females (F_3) were then backcrossed to males of either the maternal or the paternal lineage. The pattern of fitness recovery in the backcrossed animals was striking. Although no fitness recovery was observed in any of the six paternal backcrosses, maternal backcrosses resulted in nearly complete recovery to maternal parental fitness in every case (Table 2.1; Figures 2.1, 2.2, and 2.3). The differential effect of paternal versus maternal backcross was significant in all six crosses for the fecundity measure and in 11 of 18 cases across all fitness measures for all crosses (three fitness measures for six crosses); maternal backcross hybrids always outperformed paternal backcross hybrids (Figures 2.1, 2.2, and 2.3).

As a measure of overall mitochondrial function, the rate of mitochondrial ATP production in intact mitochondria isolated from parental lineages, F_3 hybrids, and maternal and paternal backcrosses was assayed. Consistent with previous results (Ellison

and Burton 2006), mitochondrial ATP production in F_3 hybrids was lower than that of the parental lineages. Rates of ATP production by mitochondria isolated from paternal backcrosses tended to be slightly higher, but not significantly different, than F_3 hybrids, while mitochondrial ATP production rate in maternal backcrosses increased significantly relative to F_3 hybrids in four of six crosses (Figure 2.4). Though the increased rate observed in maternal backcrosses was generally greater in magnitude than that in paternal backcrosses, this difference was not significant (Table 2.1), likely due to the partial recovery of mitochondrial ATP production rate in paternal backcross animals (Figure 2.4). Importantly, while both fitness and mitochondrial ATP synthetic capacity recovered in maternal backcross hybrids, they show slightly different patterns of recovery.

Discussion

Comparisons of reciprocal crosses show that fitness recovery in *T. californicus* hybrids was not achieved by restoring a full nuclear genetic complement alone. The backcross experimental design generates four unique nuclear and mitochondrial genotypes. Reciprocal F_3 hybrid pairs have, on average, identical nuclear genetic composition prior to being backcrossed, but different maternally derived mtDNA. Hence, the maternal backcross (hybrid female x maternal population male) offspring of one interpopulation hybrid has, on average, the same nuclear genetic composition as the paternal backcross (hybrid female x paternal population male) offspring of the reciprocal interpopulation hybrid. The genetic differences between the maternal and paternal backcrosses are then restricted to cytoplasmic genetic elements such as the mtDNA. Two caveats with this design are: (1) although the nuclear genetic composition of reciprocals is expected to be identical; any strong selection in the F_1 and F_2 generations may result in deviations from this expectation, and (2) although identical in expected genetic composition, gene expression in hybrids may be biased (Landry et al 2007; Ortíz-Barrientos et al 2007). Despite identical nuclear genetic composition, only the maternal backcross offspring were found to have wildtype fitness. Remarkably, fitness recovery was essentially complete in every maternal backcross, while fitness recovery was absent in paternal backcrosses. Although previous studies have shown that nuclear-mitochondrial interactions can impact enzyme activities and the relative fitnesses of different hybrid genotypes, the results presented here are the first clear demonstration that nuclear-cytoplasmic interactions play a dominant role in hybrid breakdown in *T. californicus*.

Rates of ATP synthesis in isolated mitochondria were measured to assess how nuclear-cytoplasmic interactions were manifested at the level of cellular physiology. Mitochondria isolated from F_3 hybrids showed significant reduction in ATP biosynthetic capacity relative to parental lineages. Mitochondrial ATP production was partially rescued in maternal backcrosses. However, in contrast to fitness measures, recovery of ATP synthesis was not complete in maternal backcrosses. Also contrasting with the fitness measures, some recovery of ATP synthesis was observed in paternal backcrosses. This pattern of recovery of mitochondrial function is likely complicated by the presence of nuclear-nuclear interactions in addition to nuclear-mitochondrial interactions. The mitochondrial ETS contains 13 mitochondrial-encoded and over 70 nuclear-encoded polypeptides (Sackton et al 2003), so it is reasonable to expect that some coadaptation of nuclear-encoded subunits may evolve within populations. A recent study of ETS complex III interactions in *T. californicus* hybrids, found evidence for nuclear-nuclear, but not nuclear-mitochondrial, interactions among three components of complex III (Willett 2006). While our data strongly suggest that nuclear-mitochondrial interactions are the primary component of interpopulation hybrid breakdown in fitness, it seems likely that interpopulation hybridization also results in disruption of nuclear-nuclear interactions within the ETS.

Maternal backcross hybrids show recovery of mitochondrial ETS function and concomitant recovery of fitness. ATP production capacity might reasonably be expected to have beneficial effects on fitness and previous work (Burton et al 2006; Ellison and Burton 2006) has suggested that mitochondrial function may, in fact, be positively correlated with fitness. These studies report such findings in the context of inbred hybrid

lines however, and the high homozygosity harbored in such lines may exaggerate fitness effects. In this study, partial recovery of mitochondrial function in paternal backcross hybrids without a corresponding recovery of fitness suggests that there is little correlation between mitochondrial ATP production rate and organismal fitness. This may be a consequence of statistical power. Maternal backcrosses were found to have fecundities approximately 20% greater than those of either F_2 and F_3 hybrids or paternal backcross hybrids across six crosses. While the fitness and ATP production rate recovery of maternal backcross hybrids was far greater than that of paternal backcross hybrids, the observed variance of our fitness measures far exceeded that of our measurements of ATP production in isolated mitochondria. Consequently, the resolution afforded by this study may have been unable to detect a partial recovery of fitness on the order of 5% to 10%, corresponding to the partial recovery of mitochondrial ATP production rate in paternal backcross hybrids. Thus, apparent differences of recovery patterns in fitness and mitochondrial ATP production do not necessarily preclude a functional relationship between these parameters.

Alternatively, differences in patterns of fitness recovery and recovery of mitochondrial ATP biosynthetic capacity in backcrosses may reflect disruption of other aspects of mitochondrial function. Mitochondria are rightfully regarded as the powerhouses of the cell due to their central role in cellular energy production in the form of ATP. However, the performance of alternative mitochondrial functions may also contribute to hybrid fitness. At the organellar level, mitochondria perform DNA replication, transcription, and translation and are involved in lipid metabolism in addition to the production of ATP (Scheffler 1999). Further, the function of

mitochondria in the cell is known to extend well beyond such metabolic and biosynthetic capacities to include metabolic regulation (Das 2006), apoptosis (Newmeyer and Ferguson-Miller 2003), antiviral response mechanisms (Seth et al 2005), and control of development and the cell cycle (Mandal et al 2005). While intuition may suggest that interaction between nuclear and mitochondrial genomes must necessarily involve protein-coding regions, and thus the mitochondrial ETS, the multifarious functions of the mitochondria suggest that impacts on other processes are also probable.

The evidence presented here strongly suggests that the interaction of nuclear and mitochondrial genomes from distinct populations may have serious consequences for mitochondrial function and fitness of hybrids. While previous studies have found evidence mitochondrial dysfunction in interspecific nucleocytoplasmic hybrids of both mammals (Schmidt et al 2001) and amphibians (Liepens and Hennen 1977), here I found that similar processes may act at the level of conspecific populations, as well. This intraspecific break is a consequence of extremely high mitochondrial divergence among *T. californicus* populations. It must be noted, however, that high levels of mitochondrial DNA divergence have been reported in a growing number of taxa, including both invertebrates (e.g., the snail *Cepaea nemoralis*,Thomaz et al 1996) and vertebrates (e.g. the freshwater fish *Galaxias maculates*, Waters and Burridge (1999), and the green python, *Morelia vidis* (Rawlings and Donnellan 2003)). Our results have important implications for conservation efforts in which anthropogenic enhancement of depleted populations might introduce nuclear and/or mitochondrial genomes that are incompatible with resident genomes. Although the dilemma posed by inbreeding depression is often a design consideration for conservation management plans, the

parallel danger of outbreeding depression is less frequently an explicit concern (Edmands 2007). Our results demonstrate not only that such oversight could have marked consequences for the long-term success of interpopulation hybrid individuals, but also that the role of the mitochondrial genome, often overlooked with regard to outbreeding depression, cannot be ignored.

The search for "speciation" or "barrier" genes has undergone a recent resurgence (see Noor and Feder 2006 for a review of several loci associated with reproductive incompatibility). However, pinpointing the ultimate origin of reproductive isolation between species can be a difficult undertaking, as many barriers have arisen between most species pairs and those most readily mapped may not represent the original causal barriers delineating species (Coyne and Orr 2004). Interpopulation hybrid breakdown represents an intermediate point between reproductively isolated biological species and a single, panmictic population. As a result, species with populations exhibiting hybrid breakdown, such as *T. californicus*, are uniquely suited to examine not only the process of species formation, but also the underlying mechanisms that drive it. Our data demonstrate that there is a significant cytoplasmic contribution to hybrid breakdown in *T. calfornicus* and that possession of a complete haploid nuclear genome and the mitochondrial genome from the same parent is sufficient to restore fitness in hybrids. Interestingly, the pattern of recovery of mitochondrial ATP production is not fully congruent with that of fitness, possibly due to the presence of nuclear-nuclear interactions or the action of alternative mitochondrial functions. I conclude that interactions between nuclear and mitochondrial genomes represent an important, and

often underappreciated, component of hybrid breakdown and species formation with potential effects in a variety of applications.

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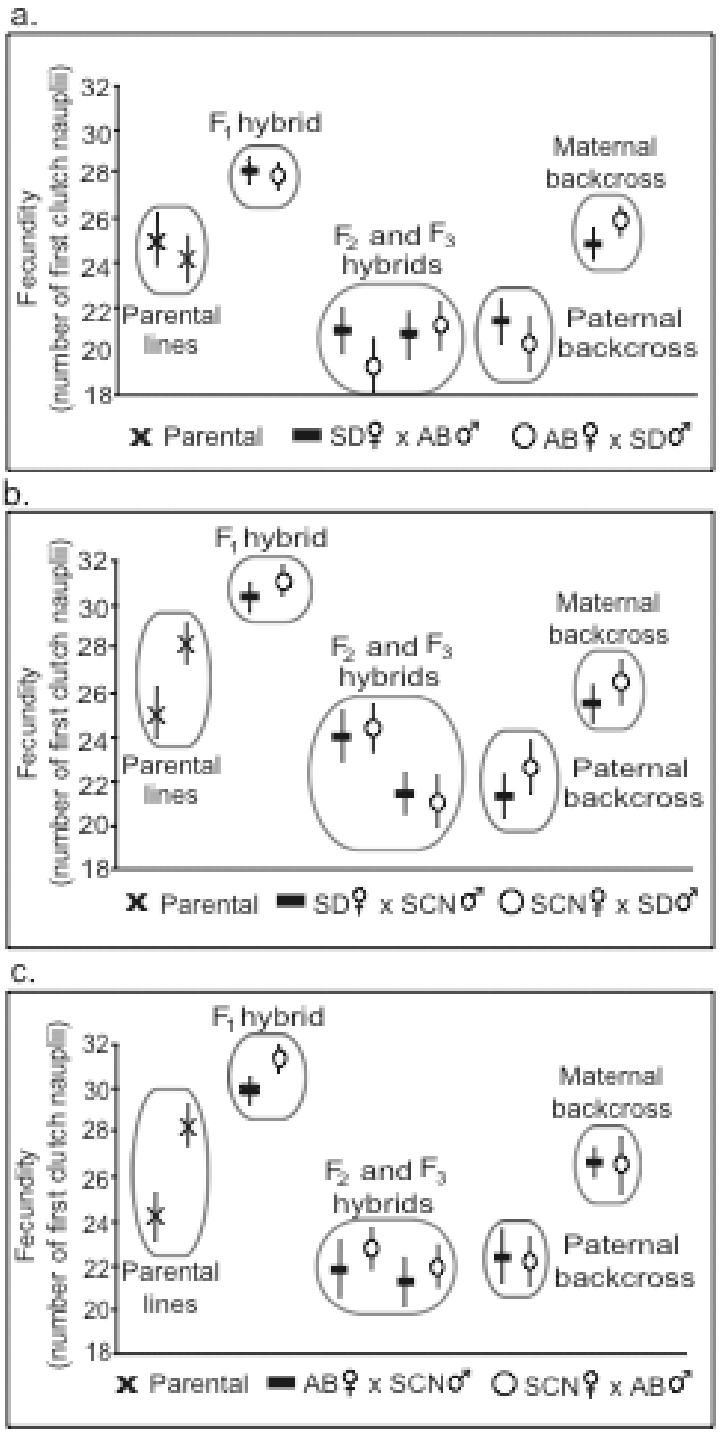

Figure 2.1: Maternal backcrossing rescues fecundity. Fecundity (mean $\pm$ 1 s.e.m.) for parental lineages, F_1, F_2, and F_3 hybrids, and maternal and paternal backcrosses for three interpopulation hybridizations and their reciprocals. Panel a = SD and AB hybrids, panel b = SD and SCN hybrids, panel c = AB and SCN hybrids. n = 10 for each point.

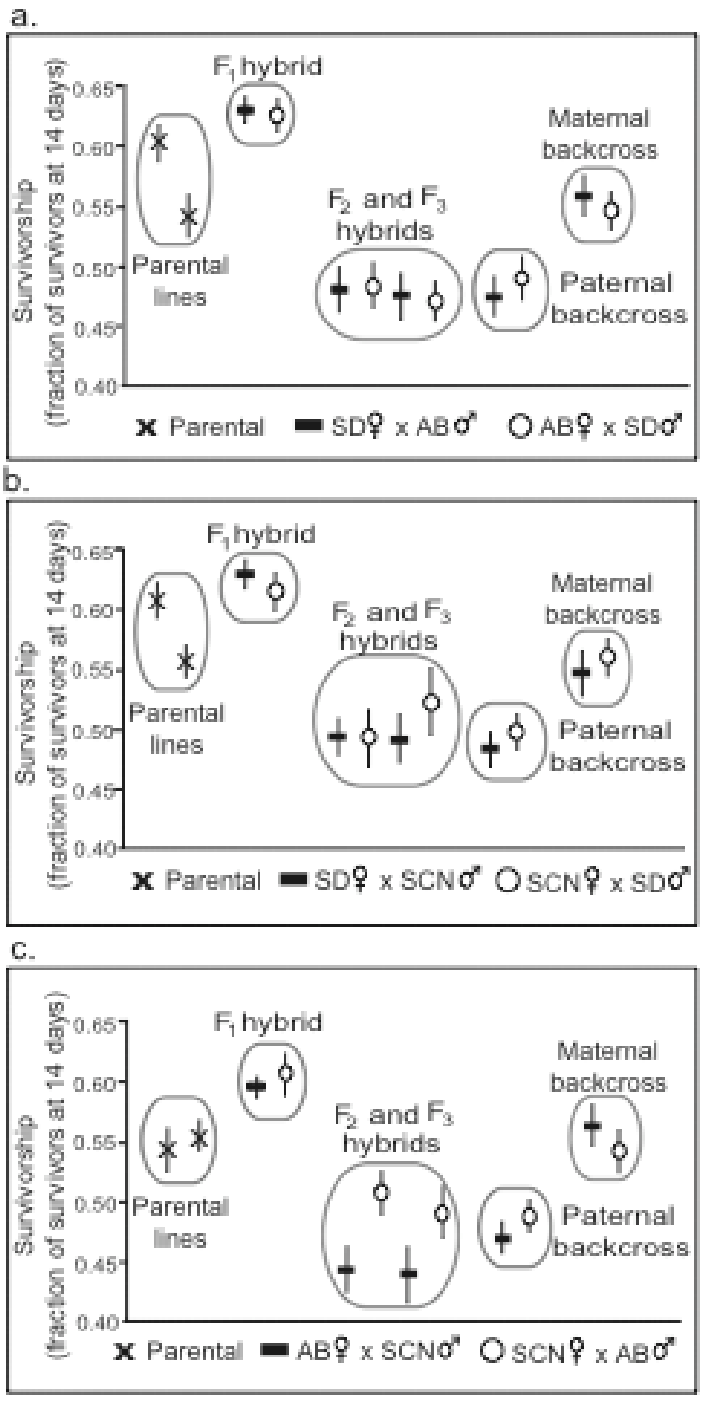

Figure 2.2: Maternal backcrossing rescues survivorship. Survivorship fractions (mean ± 1 s.e.m.) for parental lineages, F_1, F_2, and F_3 hybrids, and maternal and paternal backcrosses for three interpopulation hybridizations and their reciprocals. Panel a = SD and AB hybrids, panel b = SD and SCN hybrids, panel c = AB and SCN hybrids. n = 10 for each point.

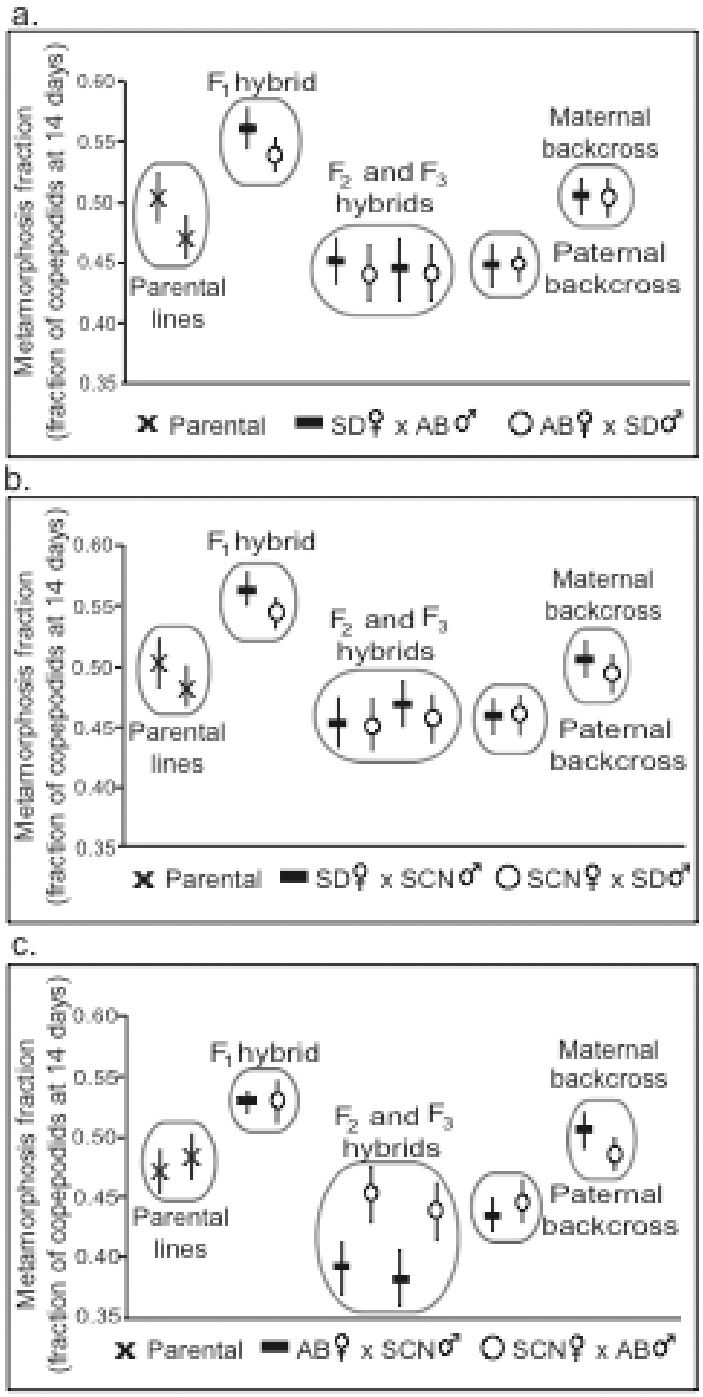

Figure 2.3: Maternal backcrossing rescues metamorphosis rate. Metamorphosis fraction (mean $\pm$ 1 s.e.m.) for parental lineages, F_1, F_2, and F_3 hybrids, and maternal and paternal backcrosses for three interpopulation hybridizations and their reciprocals. Panel a = SD and AB hybrids, panel b = SD and SCN hybrids, panel c = AB and SCN hybrids. n = 10 for each point.

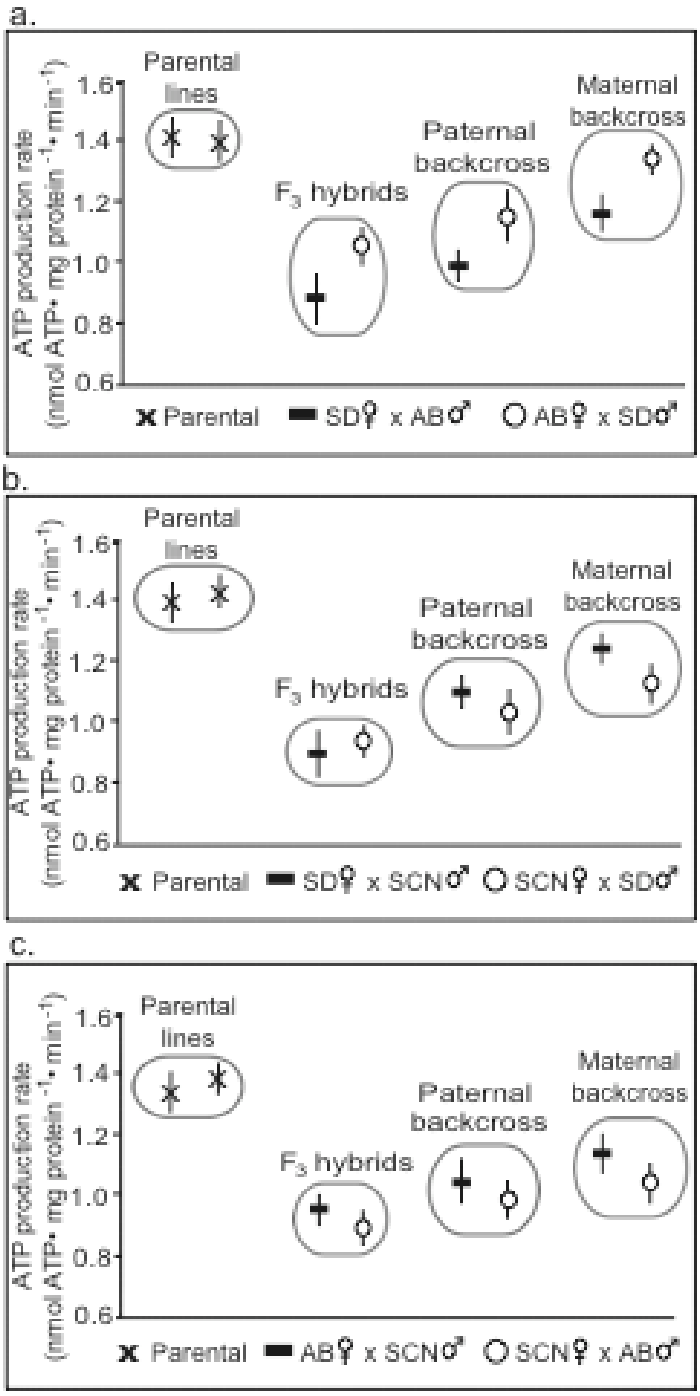

Figure 2.4: Mitochondrial ATP production rate is partially rescued in all backcrosses. Mitochondrial ATP production rate (mean $\pm$ 1 s.e.m.) for parental lineages, F_3 hybrids, and maternal and paternal backcrosses for three interpopulation hybridizations and their reciprocals. Panel a = SD and AB hybrids, panel b = SD and SCN hybrids, panel c = AB and SCN hybrids. n = 10 for each point.

Table 2.1: Comparison of hybrid and hybrid backcross fitnesses and mitochondrial ATP production for six interpopulation crosses. Significance values are given for ANOVA addressing five hypotheses: (1) does the maternal backcross outperform hybrid animals? (F_3 hybrids vs maternal backcross, Bm), (2) does the paternal backcross outperform hybrid animals? (F_3 hybrids vs paternal back cross, Bp), (3) do maternal and paternal backcrosses result in differential fitnesses? (Bm vs Bp), (4) is fitness recovered to the level of the maternal parent in the hybrid maternal backcrosses? (maternal backcross, Bm, vs maternal parent P), and (5) is fitness recovered to the level of the maternal parent in the hybrid paternal backcross? (paternal backcross, Bp, vs maternal parent, P). n = 10 for each category of data. (*) denotes significance at alpha = 0.05

Maternal parent:		SD	AB	SD	SCN	AB	SCN
Paternal parent:		AB	SD	SCN	SD	SCN	AB
	F_3 vs Bm	0.0346*	0.0194*	0.0757	0.0124*	0.0054*	0.0375*
	F_3 vs Bp	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Fecundity	Bm vs Bp	0.0281*	0.0044*	0.0638	0.1482	0.0474*	0.0016*
	Bm vs P	1.0000	1.0000	1.0000	1.0000	0.6164	1.0000
	Bp vs P	0.0797	0.0909	0.0757	0.0050*	1.0000	0.0023*
	F_3 vs Bm	0.0081*	0.0148*	0.2018	1.0000	<0.0001*	0.5759
	F_3 vs Bp	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Survivorship	Bm vs Bp	0.0074*	0.0188*	0.0627	0.5296	0.0020*	0.1123
	Bm vs P	0.2282	1.0000	0.0820	1.0000	1.0000	1.0000
	Bp vs P	<0.0001*	0.1473	<0.0001*	0.5849	0.0168*	0.0270*
	F_3 vs Bm	0.1408*	0.0824	0.6661	0.7253	<0.0001*	0.2961
	F_3 vs Bp	1.0000	1.0000	1.0000	1.0000	0.2260	1.0000
Metamorphosis	Bm vs Bp	0.2255	0.1961	0.3702	0.8992	0.0361*	0.4835
	Bm vs P	1.0000	1.0000	1.0000	1.0000	0.9833	1.0000
	Bp vs P	0.248	1.0000	0.5137	1.0000	0.8572	0.6036
	F_3 vs Bm	0.0167*	0.0137*	0.0006*	0.1533	0.0002*	0.5677
	F_3 vs Bp	1.0000	1.0000	0.0721	1.0000	0.5031	1.0000
ATP production	Bm vs Bp	0.3035	0.2242	0.5581	1.0000	0.2086	1.0000
	Bm vs P	0.0521	1.0000	0.1717	0.0050*	0.3595	0.0001*
	Bp vs P	0.0002*	0.0401*	0.0018*	0.0002*	0.0012*	<0.0001*

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CHAPTER III

Disruption of mitochondrial function in interpopulation hybrids of *Tigriopus californicus*

Abstract

Electron transport system (ETS) function in mitochondria is essential for the aerobic production of energy. Because ETS function requires extensive interactions between mitochondrial and nuclear gene products, coadaptation between mitochondrial and nuclear genomes may evolve within populations. Hybridization between allopatric populations may then expose functional incompatibilities between genomes that have not coevolved. The intertidal copepod *Tigriopus californicus* has high levels of nucleotide divergence among populations at mitochondrial loci and suffers F_2 hybrid breakdown in interpopulation hybrids. I hypothesize that hybridization results in incompatibilities among subunits in ETS enzyme complexes and that these incompatibilities result in diminished mitochondrial function and fitness. To test this hypothesis, I measured fitness, mitochondrial function, and ETS enzyme activity in inbred recombinant hybrid lines of *T. californicus*. I find that (1) both fitness and mitochondrial function are reduced in hybrid lines, (2) only those ETS enzymes with both nuclear and mitochondrial subunits show a loss of activity in hybrid lines, and (3) positive relationships exists between ETS enzyme activity and mitochondrial function and between mitochondrial function and fitness. I also present evidence that hybrid lines harboring mtDNA and mitochondrial RNA polymerase from the same parental source population have higher fitness than those with mtDNA and mtRPOL from different populations, suggesting that mitochondrial gene regulation may play a role in disruption of mitochondrial performance and fitness of hybrids. These results suggest that disruption of coadaptation between nuclear and mitochondrial genes contributes to the phenomenon of hybrid breakdown.

Introduction

Cellular metabolic energy production is critically dependent on nuclear-mitochondrial interactions. Only 13 polypeptides are encoded by the mitochondrial genome and all function as integral subunits in the major enzyme complexes of the mitochondrial electron transport system (ETS). Over 95% of proteins present in the mitochondria are encoded in the nuclear genome; approximately 70 of these polypeptides comprise subunits of the ETS (Grossman et al. 2004; Rand et al. 2004) and thus must interact directly with subunits encoded in the mitochondrial genome. The efficacy of these interactions, in turn, influences overall mitochondrial function and generates strong selection for positive epistatic interactions between nuclear and mitochondrial genes.

Substantial effort has been invested in exploring the nature of nuclear-mitochondrial interactions in both primate and murid xenomitochondrial cybrid cell lines. This technique allows the introduction of foreign mitochondria into amitochondrial cell lines to generate interspecific hybrids. One such study by Kenyon and Moraes (1997) found a decrease in complex I activity in human xenomitochondrial cybrids harboring either chimpanzee or gorilla mitochondria, consistent with intraspecific coadaptation between nuclear and mitochondrial encoded subunits in complex I. Later studies have also indicated a strong effect on complex IV (Barrientos et al. 2000). Because mitochondrial DNA typically evolves more rapidly than nuclear DNA (Brown et al. 1979; Ballard and Whitlock 2004), Dey et al. (2000) proposed that divergent taxa are potentially predisposed to intrinsic incompatibility between nuclear and mitochondrial gene products. Recently, McKenzie et al. (2003) tested this hypothesis using xenocybrid *Mus* cell lines with murid mitochondrial types ranging from congenerics to *Otomys* and

found that activities of complexes III and IV were strikingly deficient in intergeneric *Rattus* and *Otomys* xenocybrids.

The intertidal copepod *Tigriopus californicus* provides an excellent model system for the study of coadaptation between nuclear and mitochondrial gene products. Strong genetic differentiation has been documented among allopatric populations along the west coast of North America using allozymes and nuclear and mitochondrial DNA sequence data; uncorrected levels of sequence divergence between populations frequently exceed 15% in the mtDNA gene COXI (Burton and Lee 1994; Burton 1998; Burton et al. 1999; Edmands 2001). Despite such extensive divergence, interpopulation crosses are capable of producing fertile offspring in the laboratory (Burton et al. 1981) and demonstrate no prezygotic isolation (Ganz and Burton 1995). Although heterosis is observed in F_1 offspring of some interpopulation crosses, F_2 hybrids consistently experience hybrid breakdown in many fitness characters, including developmental time (Burton 1990), survivorship, and reproductive success (Edmands 1999).

In addition to coadaptation between nuclear genes, multiple lines of evidence suggest extensive nuclear-mitochondrial coadaptation within genetically isolated *T. californicus* populations. Edmands and Burton (1999) found reduced enzyme activity of cytochrome c oxidase (COX) in backcrossed hybrids. Repeated backcrossing effectively introgressed the nuclear genes from one population onto the cytoplasmic, and thereby mitochondrial, background of another population; the subsequent loss of COX activity could be attributed in part to nuclear-mitochondrial interactions. Rawson and Burton (2002) provide more direct evidence for the coadaptation of cytochrome c (CYC, encoded in the nucleus) and COX (partially encoded in mitochondria) in *T. californicus*.

Activity of COX from a San Diego population was found to be significantly higher when paired with San Diego-derived CYC than with CYC derived from a Santa Cruz population; similarly Santa Cruz derived COX had maximal activity when paired with Santa Cruz derived CYC. Willett and Burton (2001) were able to show that F_2 hybrid animals having a CYC genotype from a different population than the cytoplasmic background suffered significant viability effects, thereby providing a link between fitness effects at the organismal level and disruption of coadapted nuclear-mitochondrial interactions.

Much of the work described above in the *T. californicus* system has focused on the COX-CYC interaction in ETS complex IV, though this is only one component of many nuclear and mitochondrial genes that may be involved in coadapted complexes. Willett and Burton (2004) used nucleotide sequence analysis of mitochondrial encoded cytochrome b and nuclear encoded rieske iron-sulfur protein and cytochrome c_1 to expand the existing work to include subunits of ETS complex III. ETS complexes I, III, and IV are all composed of both nuclear and mitochondrial encoded subunits, while complex II is wholly nuclear-encoded (see Figure 3.1) (Saraste 1999). Complex II therefore provides an interesting internal control by which to examine the strength of nuclear-mitochondrial interaction in hybrid animals, as activity of this complex is expected to be independent of mitochondrial background while complexes I, III, and IV may experience decreased activity due to segregation of coadapted nuclear and mitochondrial proteins.

An additional nuclear-mitochondrial interaction that remains largely unexplored in *T. californicus* concerns transcription of the mitochondrial genome. Mitochondrial

RNA polymerase is encoded by a nuclear gene and binds a promoter region present in the D-loop of the mitochondrial genome to initiate transcription of mtDNA (Nam and Kang 2001; Karlok et al. 2004). Further, the binding specificity of mitochondrial RNA polymerase to the appropriate promoter(s) is mediated by the polymerase itself (Gaspari et al. 2004; Matsunaga and Jaehning 2004). Therefore, for the mitochondrial RNA polymerase to successfully bind the promoter region, there must be an interaction between a nuclear-encoded gene product and the mitochondrial genome. A mismatch between mitochondrial RNA polymerase and mitochondrial genotypes in hybrid animals may result in a diminished ability to transcribe the mitochondrial gene products necessary for the function of the ETS. In turn, this would be expected to result in a decline in overall mitochondrial function.

In this study, I measured the activity of each of the major ETS enzyme complexes as well as *in vitro* ATP production by mitochondria, fecundity, survivorship, and metamorphosis rate for a number of inbred recombinant hybrid lines derived from a series of interpopulation crosses of *T. californicus* and inbred parental control lines. Additionally, I determine the mitochondrial RNA polymerase genotype for each of the inbred recombinant hybrid lines and compare the mitochondrial function of matched versus mismatched genotypes. I use these data to examine (1) functional coadaptation between nuclear and mitochondrial encoded gene products and (2) the effect of mismatched mitochondrial RNA polymerase and mitochondrial genotypes in hybrids and finally ask whether this sort of intrinsic coadaptation can lead to hybrid breakdown in interpopulation hybrids.

Materials and Methods

Generation of Inbred Recombinant Lineages

Tigriopus californicus samples were collected from four sites along the western coast of North America ranging from central California to northern Baja California, Mexico. These locations were Santa Cruz, CA (SCN: 36°57'N, 122°03'W, collected October 2002), Abalone Cove, Palos Verdes Peninsula, CA (AB: 33°44'N, 118°22'W, collected May 2003), San Diego, CA (SD: 32°45'N, 117°15'W, collected October 2003), and Punta Morro, Baja California (PM: 31°52'N, 116°40'W, collected May 2003). Stock cultures of these animals were kept in 500 ml beakers containing 200 ml seawater at 20°C and ground, dried *Spirulina* algae at 0.2 mg l^{-1} seawater. All subsequent interpopulation and control crosses were performed in 100 mm diameter Petri dishes containing identical water, temperature, and food conditions and each generation was transferred to fresh culture.

T. californicus females mate only once and mature males exhibit mate guarding behavior by clasping virgin females with their antennae until the females are reproductively mature (Egloff 1967, Vittor 1971, Burton 1985). Clasped pairs were removed from cultures and teased apart using a fine needle to obtain separate males and virgin females from each of the populations. These animals were then experimentally crossed to generate intrapopulation parental controls (e.g. SCN x SCN) as well as interpopulation hybrid animals (e.g. SD x SCN). Crosses were performed with four replicates, each containing 5 males from one population and 5 virgin females from another. For all crosses, adult males were removed from the cultures when females developed egg sacs and adult females were removed at the first appearance of copepodid

(juvenile copepod) stage individuals and placed in a fresh culture dish to maintain discrete generations. The resulting F_1 individuals were crossed to other F_1 individuals from a replicate of the same cross; crosses were similarly performed using F_2 individuals from a different replicate of the same cross. This constituted a no-inbreeding strategy through the first two generations of hybrids. As F_3 females with egg sacs appeared, they were isolated in individual dishes and, beginning with F_4 individuals, only full sib matings were allowed for five generations, with each generation within each line being transferred to a fresh culture dish. A total of 128 intrapopulation control lines and 113 interpopulation hybrid lines were initially generated in this manner including control lines from all four experimental populations and hybrid lines comprising all possible crosses and their reciprocals . Enzymatic assays required a minimum of 60 adult copepods; only 52 lines produced sufficient numbers of animals (27 control lines plus 25 hybrid lines) and only these were used for subsequent analyses. For advanced generations beyond F_9, adult individuals were not removed from the cultures and discrete generations were no longer maintained, though salinity and water quality were continually monitored. Mitochondrial origin could be traced through each generation via the maternal lineage.

Measurement of Life History Characteristics

Data on hatching number, survivorship fraction, and metamorphosis fraction were collected for each of the lines assayed at the F_{10} (seventh inbred recombinant hybrid generation) generation. These parameters were measured following Edmands and Deimler (2004). Individual females with egg sacs were placed in 6-well plates and monitored daily; the female was promptly removed from the dish following the hatching

of the first clutch to eliminate the possibility of counting nauplii from multiple clutches. Nauplii from the first clutch were enumerated under a dissecting scope immediately following hatching to determine the hatching number (fecundity) and transferred to a new dish to facilitate accurate measurement. Survivorship and metamorphosis were evaluated 14 days after the first appearance of nauplii. Survivorship fraction was calculated as the fraction of individuals alive 14 days after hatching relative to the number of original hatching nauplii. Metamorphosis fraction was calculated as the number of copepodids present divided by the total number of survivors.

Isolation of Mitochondrial Fraction

Protocol for isolation of mitochondria was modified from the Sigma Mitochondrial Isolation Kit (Sigma, St Louis, MO). Mitochondria were isolated from either 20 or 40 adult *T. californicus* individuals for the purpose of evaluating either whole-mitochondria ATP production or mitochondrial electron transport system (ETS) enzyme activity and citrate synthase activity, respectively. Animals were individually transferred from culture to filter paper, then placed in a 2 ml microcentrifuge tube containing 500 µl filtered seawater (0.22 µm pore size, Millipore Corp, Billerica, MA). All subsequent steps were performed on ice and all reagents were kept ice-cold during the isolation process.

Seawater was aspirated from the tube, being careful not to remove any animals. Each sample was then washed briefly with 200 µl mitochondrial isolation buffer (IB; 10 mM MOPS, pH 7.5, 55 mM KCl, 500 µM EGTA) (unless otherwise indicated, all reagents are from Sigma, St Louis, MO), before again aspirating all liquid. Samples were

then incubated for 3 min in 1ml IB containing 0.25 mg ml^{-1} trypsin. This solution was aspirated and replaced with 800 µl IB solution containing 0.25 mg ml^{-1} trypsin and incubated for 10 min on ice. At the end of the incubation, 200 µl of 50 mg ml^{-1} albumin solution was added. This solution was once again aspirated before the addition of 800 µl IB and homogenization in the microcentrifuge tube using a glass pestle. Nuclei and other cellular debris were pelleted out of solution by centrifugation at 600 x *g* for 5 min at 4°C and the supernatant was subsequently transferred to a fresh microcentrifuge tube. The mitochondrial fraction was obtained by centrifugation at 11,000 x *g* for 10 min at 4°C. The supernatant was discarded and the pelleted mitochondria were resuspended in 45 µl of either IB (for ATP production assays) or storage buffer (5 mM HEPES, pH 7.5, 125 mM sucrose, 0.5 mM ATP, 40 µM ADP, 2.5 mM sodium succinate, 1 mM K_2HPO_4, and 0.5 mM DTT). Mitochondrial membrane integrity was checked using a JC-1 assay (after Salvioli et al 1997) before running assays for ATP production or storage at -80°C for later evaluation of specific ETS complex activity. Out of 108 mitochondrial isolations, 4 were discarded due to low JC-1 fluorescence.

A 5 µl aliquot of each mitochondrial suspension was removed and frozen at -80°C for protein quantfication using the NanoOrange® Protein Quantification Kit (Molecular Probes, Eugene, OR). Fluorescence measurements were made in 96-well plate format on a Fluoroskan Ascent FL (Thermo Labsystems, Franklin, MA).

In vitro measurement of ATP production

Mitochondrial samples for measuring ATP production capacity were isolated from F_9 (sixth inbred recombinant hybrid generation) individuals. ATP production was

measured using the CellTiter-Glo ATP Quantification Kit (Promega, Madison, WI), a luminescence based assay of ATP concentration in solution. For each sample, the mitochondrial suspension was divided into two 20 µl aliquots. Substrate solution (5 µl, 1 mM ADP, 9 mM pyruvate, 4 mM malate in 1X IB) was added to one and 5 µl buffer solution (IB) to the other to serve as a blank. These solutions were incubated for 2 hours at 25°C to allow for ATP production. CellTiter-Glo Reagent (25 µl) was then added to each sample, the samples were loaded into half-area 96-well plates, and assayed immediately on a Fluoroskan Ascent FL plate reader. Preliminary studies found that ATP accumulation in our mitochondrial preparations continued at a linear rate for three hours; the two hour incubation was selected to be certain rates were measured within this linear duration of the reaction. ATP production was calculated by subtracting the reading obtained from the suspension containing only buffer from that of the suspension containing substrate solution; in addition, each of these values were independently corrected for background luminescence. Finally, all values were normalized to protein content.

Measurement of Electron Transport System Enzyme Complex Activity

Mitochondrial samples used for enzyme activity measures were isolated from F_8 (fifth inbred recombinant hybrid generation) individuals, were suspended in storage buffer, and frozen at -80°C prior to being assayed. The freeze-thaw process permeabilized the mitochondrial membranes allowing assay of ETS enzyme activities. Separate assays measured activity of Complex I, Complex II, Complex IV, the sum of Complexes I and III, and the sum of Complexes II and III. Measurement of the latter two

values permitted the estimation of activity in ETS complex III. Enzyme assays used were modified from Trounce et al. (1996), with the exception of the complex IV assay, which was modified from Rawson and Burton (2002). Spectrophotometric measurements for all assays were taken with 50 µl volumes in 384-well plate format on a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA) at 25°C. Each assay was based on samples consisting of 1.5 ng mitochondrial protein.

Complex I activity was measured in a buffer containing 250 mM sucrose , 1.0 mM EDTA, 50 mM Tris-HCl (pH 7.4), 1.0 µM decylubiquinone, 2.0 mM KCN and mitochondrial protein. The reaction was initiated by the addition of 50 µM NADH and monitored for 1 min at 272 nm minus 247 nm, assuming an extinction coefficient of 8 mM^{-1} cm^{-1} for decylubiquinone. Prior to measuring complex II activity, a solution of 50 mM K_2HPO_4 , 20 mM succinate , and mitochondrial protein was incubated for 10 min at room temperature. Following this incubation, 2 µg/ml antimycin A, 2 µg/ml rotenone, 2 mM KCN, and 2 µM DCPIP were added and a blank reading was recorded. The reaction was initiated by the addition of 50 µM decylubiquinone and monitored for 3 min at 600 nm minus 750 nm, assuming an extinction coefficient of 19.1 mM^{-1} cm^{-1} for DCPIP.

Complex IV activity was measured in a buffer containing 10 mM K_2HPO_4 , pH 7.4, and 20 µM reduced CYC. The reaction was initiated by the addition of mitochondrial protein and monitored for 3 min at 550 nm minus 540 nm, assuming an extinction coefficient of 19.0 mM^{-1} cm^{-1} for reduced horse heart CYC. Reduced CYC for the complex IV assay was prepared as follows: 100 mg/ml horse heart CYC (USB, Cleveland, OH) in 10 mM K_2HPO_4, pH 7.4, was mixed with an equal volume of 0.1 M L-ascorbate. Ascorbate was removed using a PD-10 desalting column (Amersham

Biosciences, Uppsala, Sweden) and the reduced CYC was collected and frozen at -80°C until use. Concentration was determined using an extinction coefficient of horse CYC of 27.8 mM^{-1} cm^{-1} at 550 nM (Rawson and Burton 2002).

The activity of complexes I and III together was measured in a buffer containing 50 mM K_2HPO_4 , pH 7.4, 80 µM oxidized CYC (USB), 0.1 mM NADH, and 2 mM KCN . The reaction was initiated by the addition of mitochondrial protein and monitored for 3 min at 550 nm minus 540 nm, assuming an extinction coefficient of 19.0 mM^{-1} cm^{-1} for horse heart CYC. Complexes II and III together were measured in a buffer containing 40 mM K_2HPO_4, pH 7.4, 0.5 mM EDTA , 20 mM succinate, 2 mM KCN , and mitochondrial protein. The reaction was initiated by the addition of 30 µM CYC and monitored for 3 min at 550 nm minus 540 nm, assuming an extinction coefficient of 19.0 mM^{-1} cm^{-1} for horse heart CYC.

Measurement of Citrate Synthase Activity

Citrate synthase is wholly nuclear encoded enzyme operating as part of the citric acid cycle, metabolically upstream of the mitochondrial ETS, and served in these experiments as a control. Citrate synthase activity was assayed following the technique of Trounce et al. (1996) and used the same mitochondrial samples as had been used for the ETS enzyme complex assays. Activity was measured in a buffer containing 0.1M Tris-HCl, pH 7.4, 0.3 mM acetyl-CoA, 0.1 mM DTNB, and 0.5 ng mitochondrial protein. The reaction was initiated by the addition of 0.5 mM oxaloacetate and monitored for 1 min at 412 nm minus 360 nm, assuming an extinction coefficient of 13.6 mM^{-1} cm^{-1} for DTNB.

Genotyping of Lines for Mitochondrial RNA Polymerase

Flowers and Burton (in prep) have found that the mitochondrial RNA polymerase (mtRPOL) gene is polymorphic across natural populations of *T. californicus*. Each inbred line was genotyped for mtRPOL using a protocol developed by J. M. Flowers (unpublished data). DNA was prepared by digesting single copepods with 25 μl proteinase-K cell-lysis buffer at 65ºC for 1 hour followed by 100ºC for 15 min. Briefly, for crosses of PM x SD, AB x SD, and SCN x SD and their reciprocal crosses, PCR amplification was performed with a final reaction volume of 25 μl containing 0.5 units *Taq* polymerase (Sigma) and 2.5 mM $MgCl_2$ and using forward primer (MTRP-gtype.1F: 5'-CGTGTTTATCCCATCCCT-3') and reverse primer (MTRP-gtype.1R: 5'-CGAAGCTATACAAGCG-3') amplified with 40 cycles of 30 sec at 95ºC, 1 min at 50ºC, and 2 min at 72ºC. These products were digested overnight using Hinf1 restriction enzymes and buffers (New England Biolabs, Cambridge, MA) and scored on 2% agarose gels following electrophoresis. For crosses of PM x SCN and AB x SCN and their reciprocal crosses PCR amplification was performed using forward primer (MTRP-Msp1.F: 5'-GCGTATGCAATATTGCCGAGG-3') and reverse primer (MTRP-Msp1.R: 5'-TCTTTGGATAGCTCCATTCAAG-3') amplified with 40 cycles of 30 sec at 95ºC, 30 sec at 54ºC, and 1 min at 72ºC. These products were digested using Msp1 restriction enzymes and buffers and scored on 2% agarose gels following electrophoresis. PM x AB crosses were genotyped by DNA sequencing following amplification using MTRP-gype.1F and MTRP-Msp1.R primers.

Statistical Analysis

All statistical analyses were performed using SPSS Graduate Pack 11.0 software (SPSS, Chicago, IL). Independent samples t-tests with equal variance not assumed were used to compare inbred recombinant hybrid lines (n=25) with inbred parental control lines (n=27) for hatching number, survivorship, metamorphosis rate, ATP production, complex I activity, complex II activity, complex III activity, complex IV activity, and citrate synthase activity. Variance among inbred hybrid and inbred parental control lines was compared using Levene's test of homogeneity of variances. Correlations between ATP production and fitness parameters were tested using Pearson's correlation. Mitochondrial RNA polymerase genotype deviations from 1:1 ratio were calculated using X^2-tests.

Results

Life history characters

Hatching number, survivorship and metamorphosis data were obtained for all inbred lines still viable at the F_{10} generation (seventh inbred recombinant hybrid generation), numbering 16 inbred parental control lines and 20 inbred recombinant hybrid lines. Data are summarized in Table 3.1. Hybrid lines were observed to have a significantly lower hatching number than parental control lines ($p < 0.001$), with a decrease of over 40% in the number of first-clutch offspring. A similar pattern was found for survivorship ($p = 0.039$), though the magnitude of the difference was less than 5%. A more striking pattern was found for metamorphosis, in which surviving individuals from hybrid lines were found, on average, to be 13.8% less likely to have metamorphosed from nauplii to copepodids after 14 days than were individuals in parental control lines ($p = 0.012$). Further, the standard error for hatching number, survivorship, and metamorphosis increased approximately by a factor of two from parental control lines to hybrid lines (1.426 to 2.763 [$p = 0.079$], 0.011 to 0.019 [$p = 0.037$], 0.020 to 0.046 [$p = 0.110$], respectively), indicating a general pattern of increased variability among hybrid lines versus among parental control lines. These observations in hybrid lines, a decrease in the mean fitness as well as an increase in variance among hybrids, are consistent with the phenomenon of F_2 hybrid breakdown.

ATP production

Rate of ATP production was measured *in vitro* for all of the lines still viable at the F_9 generation (sixth inbred recombinant hybrid generation), numbering 20 inbred parental

control lines and 18 inbred recombinant hybrid lines. Results are summarized in Table 3.1. Hybrid lines were observed to have a highly significant decline in rate of *in vitro* ATP production, with a 32% reduction in activity relative to the values observed for parental control lines ($p = 0.001$). Further, the variance among the hybrid lines was again greater than that among the parental control lines, though this was not significant ($p=0.509$).

Correlations were calculated to assess the relationship between the *in vitro* rate of ATP production and the measures of fitness described above, hatching number survivorship, and metamorphosis. The results of these are summarized in Figure 3.2. Slopes were positive for all correlations between ATP production rate and fitness parameters, though only the relationship between ATP production and survivorship was significant ($p = 0.037$). This indicates a generally positive relationship between mitochondrial function and these measures of fitness.

Enzyme assays

All enzyme assays were completed using mitochondria isolated from inbred lines in the F_8 generation (fifth inbred recombinant hybrid generation). In each case, 25 hybrid lines were assayed and 27 parental control lines were assayed. Results are summarized in Table 3.1. Significant declines in mean activity were observed in hybrid lines for complex I, complex III, and complex IV, while there was no significant difference in mean activity of complex II or citrate synthase between parental control lines and hybrid lines. In the case of complex I, mean enzyme activity was observed to decrease by 30% in hybrid lines relative to parental control lines ($p <0.001$). In contrast, mean complex II

enzyme activity in hybrid lines increased relative to parental control lines by 2%, though the increase was not significant ($p = 0.735$). Mean enzyme activities for complexes III and IV decreased by 31% and 14%, respectively, in hybrid lines ($p < 0.001$ in both cases) and mean activity of citrate synthase decreased 11% in hybrid lines, though this was not significant ($p = 0.112$). In each of the five categories of enzyme assays, the standard error among hybrid lines was greater than that of parental control lines, generally on the order of a two-fold increase in magnitude ($p=0.002$ for complex I; $p=0.008$ for complex II; $p=0.001$ for complex III; $p=0.046$ for complex IV; $p=0.072$ for citrate synthase). In a manner similar to the fitness and ATP production rate data described above, this follows the established pattern of F_2 hybrid breakdown. Mean ATP production and enzyme activities for hybrid lines normalized to parental control lines are shown in Figure 3.3.

Correlations were drawn between ETS enzyme complex activity and ATP production rate data to evaluate the relationships between enzyme activity and function of whole mitochondria *in vitro*. Results are shown in Figure 3.4. Slopes were positive for correlations between ATP production rate and complex I, complex II, complex III, and complex IV, indicating a generally positive relationship between activity of ETS enzyme complexes and output of ATP by the mitochondria. In the case of complexes III and IV, these relationships were found to be significant ($p = 0.003$ and $p = 0.011$, respectively).

Mitochondrial RNA Polymerase Genotyping

A total of 23 hybrid lines were genotyped for the mitochondrial RNA polymerase locus. As expected from their inbred histories, 22 of the lines were homozygous. Of these, four were found to have a mismatch between the source population for the mtDNA

(inferred from maternal inheritance) and the source population of the mtRNA polymerase (determined by genotyping); the remaining 18 contained matched mtRNA polymerase and mtDNA genotypes. One additional line was found to be heterozygous at the mtRNA polymerase locus and was removed from the analysis. Deviation from an expected 1:1 ratio of matches to mismatches was found to be significant ($X^2 = 8.909$, df = 1, p = 0.003). Mismatched hybrid lines performed more poorly than matched hybrid lines for measures of hatching number, survivorship, metamorphosis, ATP production, complex III and complex IV activities, though this effect was only significant for ATP production rate (p = 0.032). Enzyme activities measured for complex I, complex II, and citrate synthase were slightly higher among mismatched hybrid lines than matched hybrid lines, though in no case was this effect significant (Figure 3.5). However, the statistical power of these tests was low because only four mismatched lines were available.

Discussion

The well-documented genetic divergence between populations of *T. californicus* and the feasibility of conducting crosses between these populations in the lab makes this an exciting system in which to investigate nuclear-mitochondrial coadaptation. Here I focus on three aspects of nuclear-mitochondrial coadaptation: (1) the extent to which interactions between nuclear and mitochondrial gene products affect the function of individual ETS components and overall mitochondrial function, (2) whether reductions in ETS activity and mitochondrial ATP production are related to reduced fitness in F_2 interpopulation hybrids, and (3) if reduced mitochondrial performance in F_2 hybrids might result in part from population specific interactions between the mtRNA polymerase and variation in the mtDNA control region.

Previous studies of F_2 hybrid breakdown in *T. californicus* have shown a high degree of variance among individuals in the of F_2 hybrid generation (Burton 1990; Edmands 1999). However, limitations in assay sensitivity dictated that measurements of *in vitro* rate of ATP production and enzymatic activity could not be made on individual animals. Our approach, then, employed inbred recombinant hybrid lines in lieu of using hybrid individuals. I allowed only full-sib matings to occur beginning with the F_3 generation, thus increasing the average homozygosity in each lineage with each generation and permitting assays on a range of hybrid genotypes. The process of inbreeding increases the homogeneity between individuals within lines, reducing the problems associated with pooling individuals for measurement. Additionally, increasing homozygosity within lines improved the probability of finding mismatched nuclear and

mitochondrial genes, as nuclear genes were driven toward fixation of either the original maternal or paternal gene copy.

For the purpose of comparing performance of inbred recombinant hybrid lines and inbred parental control lines, I grouped all hybrid lines together and all control lines together, though these lines were comprised of crosses derived from several different parental populations. Previous studies using the *T. californicus* system have shown negative nuclear-cytoplasmic interactions in some, but not all, interpopulation crosses (e.g. Edmands and Burton 1999 when measuring COX activity in backcrossed individuals). Pooling of data from hybrid lines during analysis is therefore likely to bias the results against finding any significant differences in performance and is a conservative approach to studying the impact of mitochondrial function on hybrid breakdown.

During generation of inbred recombinant hybrid lines as well as inbred parental control lines, the exposure of deleterious recessive alleles to selection is assumed to take a toll on the viability of many lines. Thus, it was important to ensure that the pattern of F_2 hybrid breakdown, observed previously in *T. californicus* F_2 hybrid individuals, was also evident among the inbred hybrid lines. To characterize this breakdown, I took measures of hatching number, survivorship, and metamorphosis. For all three characteristics, a general trend toward loss of fitness was observed in hybrid lines. The trend toward reductions in performance in hybrid lines was also clear at the sub-cellular level: *in vitro* rate of ATP production was markedly reduced among mitochondria derived from inbred hybrid lines relative to comparably inbred parental lines. To extend these findings to the level of the protein function, I evaluated the activity of each of the ETS enzyme

complexes individually. Cytonuclear coadaptation between COX and CYC has been extensively studied in *T. californicus* (e.g. Edmands and Burton 1999; Rawson and Burton 2002) and this interaction, between CYC and complex IV of the ETS, has also been shown to have viability effects (Willet and Burton 2001). A similar dynamic in which nuclear and mitochondrial gene products must interact can also be found within complex IV, which itself contains both nuclear and mitochondrial encoded subunits, as well as complexes I and III, which harbor a similar mosaic (nuclear-mitochondrial) makeup. All three of these enzyme complexes showed a significant decline in activity in hybrid lines when compared to parental control lines. In contrast, the activities of complex II and citrate synthase, both entirely nuclear encoded, showed no loss of activity in the same hybrid lines.

Previous studies of complex IV activity in *T. californicus* have yielded results consistent with either nuclear-nuclear interactions (there are 10 nuclear encoded subunits of complex IV) or nuclear-mitochondrial interactions, though models of nuclear-mitochondrial interactions provide the only significant fit to the data. (Edmands and Burton 1999). While the results presented here may be influenced by nuclear-nuclear interactions in addition to nuclear-mitochondrial interactions, the latter are likely predominant. In support of this inference, Willett and Burton (2001) showed that fitnesses of cytochrome c variants differed in reciprocal crosses, suggesting interaction between this nuclear gene and a cytoplasmic factor (presumably mtDNA). Willett and Burton (2004) found a 5.8 to 9.7-fold reduction in nonsynonymous substitution rates of nuclear encoded components of complex III relative to mitochondrial components of the same enzyme complex in *T. californicus* and subsequent sequencing of other nuclear-

encoded components of ETS enzyme complexes have revealed a minimal number of substitutions at several loci (J.S. Harrison, unpublished data). While the small size of complex II relative to other ETS enzyme complexes may minimize the number of suboptimal nuclear-nuclear interactions in hybrid animals at complex II relative to the larger enzyme complexes of the ETS, the majority of negative epistatic interactions in the ETS in hybrids will likely involve a substitution in a mitochondrial gene product due to the greatly reduced non-synonymous substitution rate at *T. californicus* nuclear versus mitochondrial loci. Though this does not eliminate the possibility of nuclear-nuclear interactions, the general pattern of decreased activity in complexes I, III, and IV (nuclear-mitochondrial mosaics), but not in complex II or citrate synthase (nuclear only) is consistent with the hypothesis of coadaptation between interacting mitochondrial and nuclear gene products within isolated populations.

Three conclusions are clear thus far: (1) hybrid lines suffer loss of fitness, (2) mitochondria isolated from hybrid lines have decreased ability to produce ATP, and (3) functional constraints generated by nuclear-mitochondrial coadaptation within populations can lead to loss of activity in those ETS enzyme complexes containing both nuclear and mitochondrial encoded subunits in interpopulation hybrid lines. The question is then whether there is a causal link between these three observations. Our data indicate a positive relationship between ETS enzyme activities and rates of ATP production. This relationship is not unexpected, as the activity of the four ETS enzyme complexes is responsible for generating the proton gradient that enables oxidative phosphorylation by ATP synthase. The correlation between ATP production and enzyme activity was only significant in the cases of complex III and complex IV, though this may be a consequence

of low statistical power, or may reflect reduced sensitivity of the ETS pathway flux to changes in complex I and complex II enzyme activities (Kacser and Burns 1981). The significant relationships between complex III and complex IV and ATP production may have resulted from greater statistical power due to greater between-line variation in activity of complex III or a rate limiting function in the pathway in complex IV, as this is the terminal step of the ETS. What is clear, both intuitively and from the data, is that increased activity of the ETS enzyme complexes is positively related to increased rate of ATP production by the mitochondria. Although intuition suggests that ATP production and fitness are likely to be correlated, exactly how such a relationship would be routinely manifested and empirically demonstrated is not obvious. Here I found positive relationships between rate of ATP production and hatching number, survivorship, and metamorphosis. Despite the fact that only the survivorship measure yields a significant relationship, there is a general trend toward a positive relationship between mitochondrial function (measured as rate of ATP production) and organismal fitness. I can, therefore, establish a putative connection between nuclear-mitochondrial coadaptation at the level of enzymes and hybrid breakdown in fitness.

An additional level of nuclear-mitochondrial interaction takes place between the mitochondrial control region (D-loop) and the nuclear encoded mitochondrial RNA polymerase. I conducted a preliminary investigation to assess whether coadaptation between the mitochondrial RNA polymerase and the mitochondrial genome existed by evaluating mitochondrial function among inbred hybrid lines having both mtRNA polymerase and mtDNA from the same parental population and those having a mismatch in which the two originated from different parental populations. Given that the process of

inbreeding is expected to result in the fixation of either parental mtRNA polymerase allele with equal probability (assuming selective neutrality), half of the inbred hybrid lines are expected to have generated a mismatch. In the 22 hybrid lines that were genotyped as homozygous at the mitochondrial RNA polymerase locus, only four were mismatches. This statistically significant deviation from the expectation suggests that mismatched genotypes are selected against in this interaction. Further evidence for this can be found when comparing the fitness, ATP production, and enzyme activity data for mitochondrial RNA polymerase matched and mismatched hybrid lines. Hatching number, survivorship, metamorphosis, ATP production rate, and activities of complexes II, III, and IV all are diminished in those lines harboring mismatches, though this effect is only significant in the case of ATP production rate (again, the small number of mismatched lines results in low statistical power for these tests). In any case, these data suggest that mitochondrial function is diminished when mtDNA and mtRNA polymerase are mismatched; consequently, selection will favor individuals with coadapted mtDNA and mtRNA polymerase.

Studies of the functional aspects of nuclear-mitochondrial coadaptation have been largely limited to distantly related taxa that require the use of manipulated mammalian cell lines to generate hybrids (e.g. McKenzie et al. 2003). This approach precludes any direct measurement of fitness effects suffered by hybrid organisms due to segregation of coadapted gene products. Our results demonstrate that nuclear-mitochondrial coadaptation within populations can generate mitochondrial dysfunction and reduction of hybrid fitness. These effects on mitochondrial function may pose an intrinsic post-zygotic isolating barrier for conspecific populations having strong genetic divergence.

Acknowledgements

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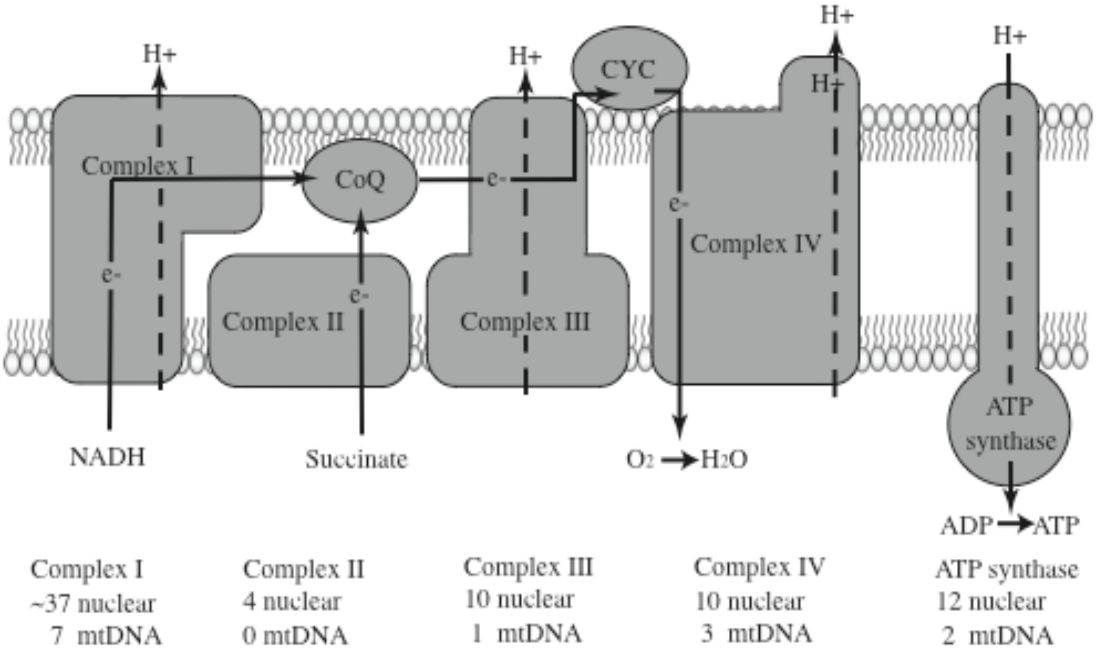
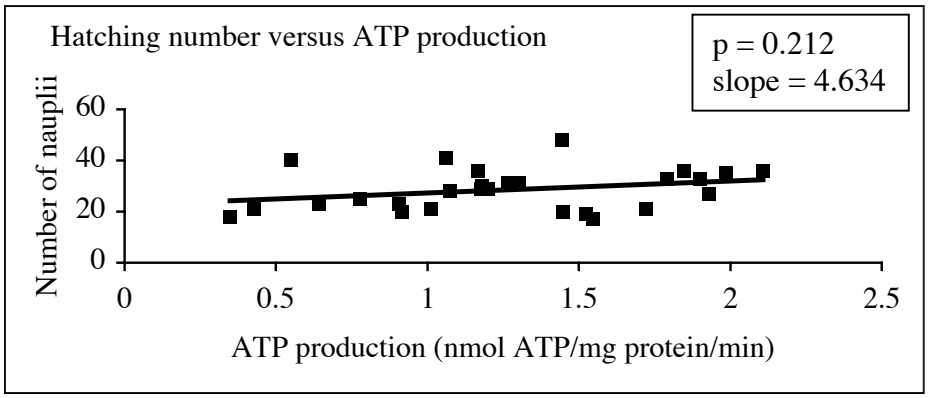


Figure 3.1: Diagram of electron transport system (ETS) embedded on the internal mitochondrial membrane. Note that complexes I, III, and IV contain both nuclear and mitochondrial encoded subunits and that complex II contains only nuclear encoded subunits.

A)



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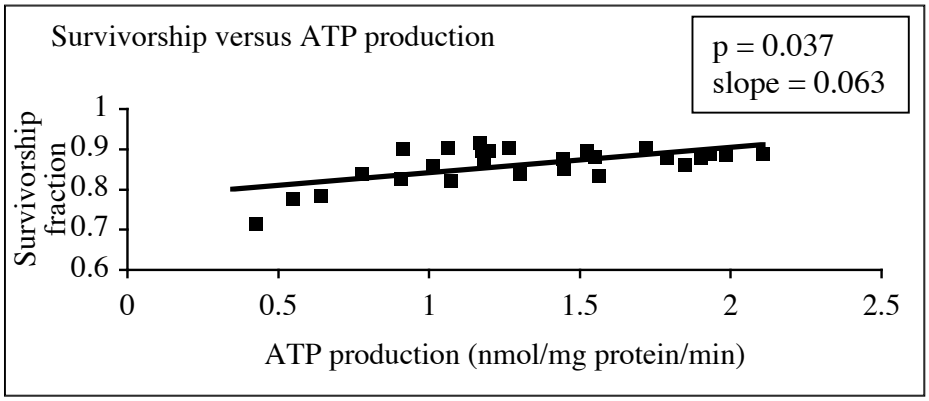

C)

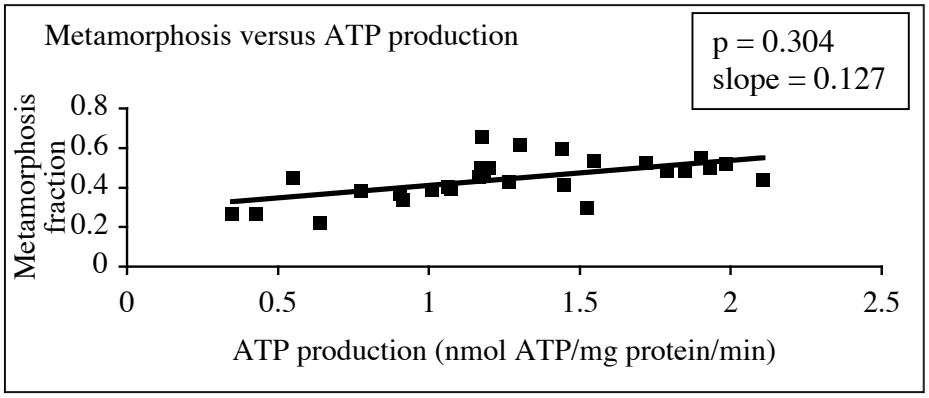

Figure 3.2: Correlations between (A) hatching number and rate of ATP production, (B) survivorship and rate of ATP production, and (C) metamorphosis and rate of ATP production. Significance based on Pearson's correlation and slopes calculated based on least-squares regression (regression lines shown on figure).

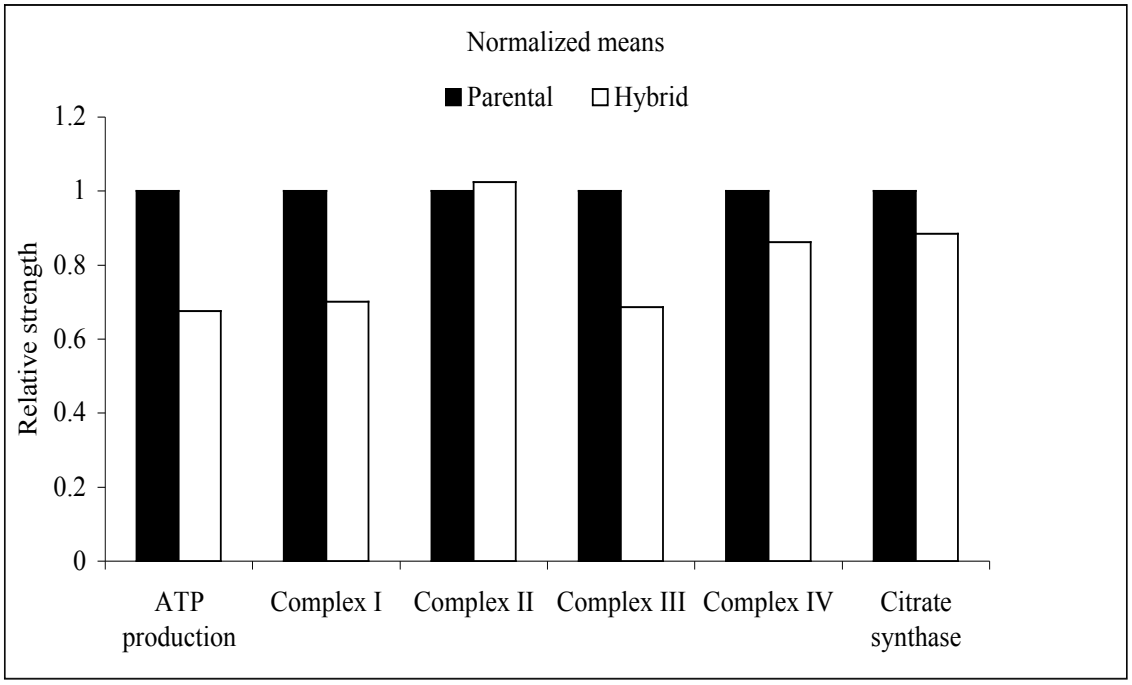

Figure 3.3: Mean values of ATP production rate and activity of complexes I, II, III, and IV, and citrate synthase for inbred parental control lines and inbred recombinant hybrid lines normalized to parental control line values. ** $p < 0.01$, *** $p < 0.0001$.

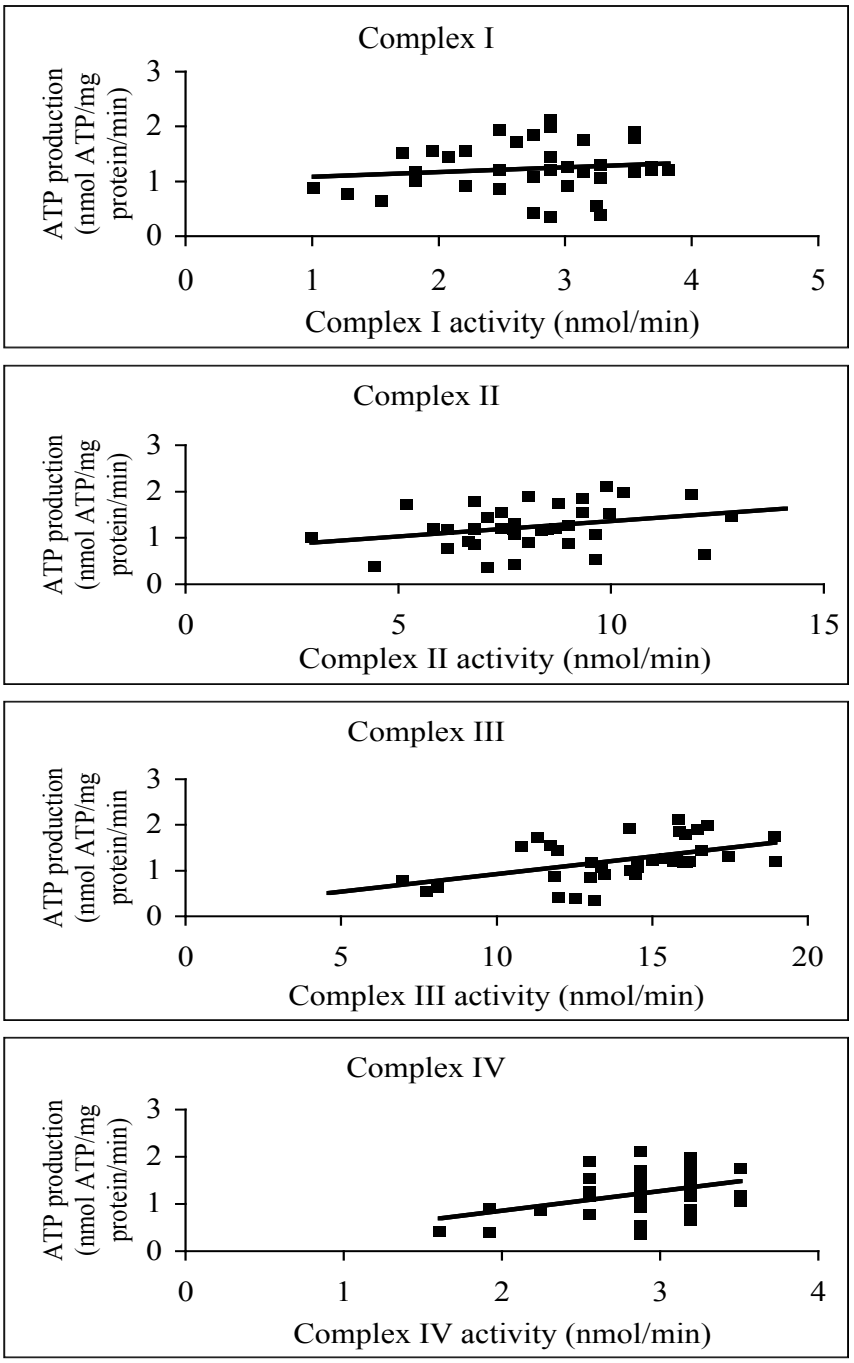

Figure 3.4: Correlations between (A) rate of ATP production and complex I activity, (B) rate of ATP production and complex II activity, (C) rate of ATP production and complex III activity, and (D) rate of ATP production and complex IV activity. Significance based on Pearson's correlation and slopes calculated based on least-squares regression (regression lines shown on figure).

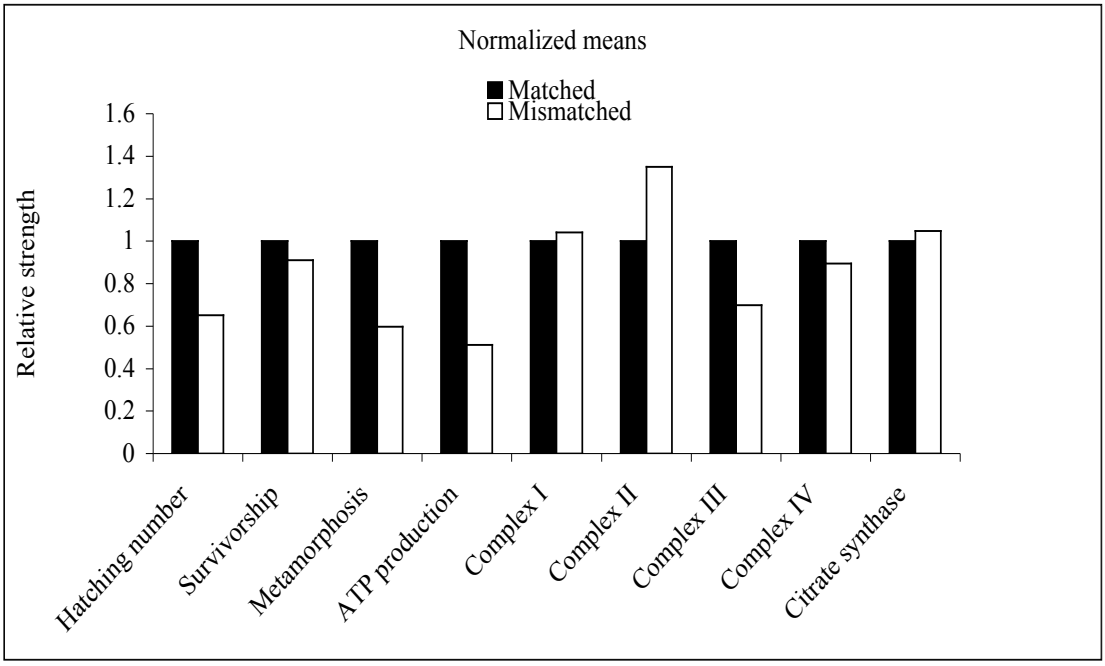

Figure 3.5: Mean values of fitness parameters, ATP production rate, and activity of complexes I, II, III, and IV, and citrate synthase for inbred recombinant hybrid lines containing mitochondrial RNA polymerase and mitochondrial genotypes derived from the same parental population (matched) and derived from different parental populations (mismatched). Data normalized to mean values for matched lines. * $p<0.05$.

Table 3.1: Means and standard error for each measure of inbred parental control lines and inbred recombinant hybrid lines. Hatching number reported as number of nauplii present in first clutch; survivorship reported as fraction of individuals surviving 14 days after hatching; metamorphosis reported as fraction of surviving individuals as copepodids 14 days after hatching; ATP production reported as nmol ATP mg protein^{-1} min^{-1}; Complexes I, II, III, and IV and citrate synthase reported as nmol substrate min^{-1}. Significance based on independent samples t-tests with equal variance not assumed to test identity of means.

	Hatching number	Survivorship	Metamorphosis	ATP production
Parental lines				
mean	33.125	0.899	0.513	1.459
(standard error)	1.429	0.011	0.02	0.078
Hybrid lines				
mean	19.05	0.851	0.377	0.9857
(standard error)	2.763	0.019	0.046	0.102
Significance	<0.001	0.039	0.012	0.001

	Complex I	Complex II	Complex III	Complex IV	Citrate synthase
Parental lines					
mean	3.2	8.217	16.017	3.15	4.433
(standard error)	0.067	0.267	0.217	0.05	0.15
Hybrid lines					
mean	2.25	8.417	11	2.717	3.933
(standard error)	0.133	0.533	0.533	0.083	0.267
Significance	<0.001	0.735	<0.001	<0.001	0.112

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CHAPTER IV

Genotype-dependent variation of mitochondrial transcriptional profile in interpopulation hybrids

Abstract

Hybridization between populations can disrupt gene expression, frequently resulting in deleterious hybrid phenotypes. Reduced fitness in interpopulation hybrids of the marine copepod *Tigriopus californicus* has been traced to interactions between the nuclear and mitochondrial genomes. Here, I determine transcript levels of four to six genes involved in the mitochondrial oxidative phosphorylation pathway for a series of parental and inbred hybrid lines using RT-qPCR. Both nuclear and mitochondrial-encoded genes are included in this analysis. Although all genes studied are upregulated under salinity stress, only expression of genes located on the mtDNA differed among lines. Because mitochondrial genes are transcribed by a dedicated, single-subunit RNA polymerase, I compare transcript levels among hybrid lines with different combinations of mitochondrial RNA polymerase and mtDNA genotypes. Lines bearing certain mtDNA-mitochondrial RNA polymerase genotypic combinations show a diminished capacity to upregulate mitochondrial genes in response to hypoosmotic stress. Effects on the transcriptional profile are cross dependent and are correlated with viability effects. I hypothesize that disruption of the mitochondrial transcriptional system in F_2 hybrids may play a central role in hybrid breakdown.

Introduction

Geographically isolated populations gradually diverge due to the combined forces of mutation, selection, and genetic drift. This genetic divergence may ultimately result in reproductive isolation and the formation of new species. During the course of this process, hybrids between populations experience reductions of fitness; typically restricted to the F_2 and later generations initially. Although the widely-cited Dobzhansky-Muller model (Dobzhansky 1936; Muller 1942) provides a general mechanism for F_2 hybrid breakdown, detailed understanding of gene-gene interactions underlying the phenomenon remain elusive. Such interactions may occur at both the structural level of protein-protein interaction and at the regulatory level, typically involving protein-nucleic acid interaction.

Expression of many genes, particularly rapidly-evolving and male-biased genes, is disrupted in hybrids and can have strongly deleterious effects (Michalak and Noor 2003; Ranz et al. 2004; Haerty and Sing 2006). Epistatic interactions are ubiquitous in all regulatory networks (Brem et al. 2005) and their dysfunction in hybrids is often envisioned as a product of altered interaction strength between transcription factors and their binding sites evolving under a Dobzhansky-Muller or compensatory evolution model (Johnson and Porter 2000; Landry et al 2007; Ortíz-Barrientos et al. 2007). However, identification of specific transcription factors, RNA polymerases, or cis-regulatory motifs underlying these observations has proven difficult due to the complexity of transcriptional networks.

No studies to date have directly addressed the impact of hybridization on the expression of mitochondrial genes, which rely on a wholly different transcriptional

system than nuclear genes. The animal mitochondrial genome typically encodes thirteen polypeptides, each of which is an integral component of the oxidative phosphorylation pathway (OXPHOS) (Scheffler 1999). Normal OXPHOS operation relies on the functional interaction and coordinated expression of nuclear and mitochondrial genes (Taylor and Turnbull 2007). In contrast to the far larger nuclear genome, the mitochondrial genome is extremely compact, with only a single region thought to contain the important regulatory features for mitochondrial gene expression (Asin-Cayuela and Gustafsson 2007). The mitochondrial genome is transcribed by a dedicated, nuclear-encoded RNA polymerase (mtRPOL) which functions independently of the transcription network of nuclear-encoded OXPHOS genes. In contrast to the large, multimeric nuclear RNA polymerase complexes, mtRPOL is a simple, phage-derived polymerase consisting of a single-subunit core polymerase, mitochondrial transcription factor A (TFAM), and one of two mitochondrial transcription factor B paralogues (TFB1M and TFB2M) (Masters et al. 1987; Tiranti et al. 1997). Additionally, mtRPOL, and not one of the transcription factors, appears to be responsible for promoter binding affinity and specificity (Gaspari et al. 2004; Matsunaga and Jaehning 2004).

Recent work has demonstrated a ubiquitous pattern of F_2 hybrid breakdown in laboratory crosses between populations of the marine copepod, *Tigriopus californicus.* (Burton 1986, 1990; Edmands 1999). Backcrossing approaches have mapped the effect to the cytoplasm and suggest that hybridization disrupts population-level cytonuclear coadaptation (Edmands and Burton 1999; Ellison and Burton 2008). In support of this hypothesis, mitochondrial function is reduced in interpopulation hybrids, as are the activities of those mitochondrial enzyme complexes requiring integration of nuclear and

mitochondrial gene products (Ellison and Burton 2006). Some of this effect may be attributed to known deleterious protein-protein interactions present in hybrids (Rawson and Burton 2002; Harrison and Burton 2006; Willett 2006). However, the pervasive nature of breakdown in all enzyme complexes containing both nuclear and mitochondrial gene products (Complexes I, III, IV, and V) and its absence in the single, wholly nuclear-encoded enzyme complex (Complex II) suggests that regulation of mtDNA transcription may play a role. Although regulatory components of this system are largely unexplored, extensive mtDNA control region variation and numerous amino-acid substitutions between population-specific alleles of mtRPOL have been found (Flowers 2005; Burton et al 2007).

Here, I investigate the impact of mtRPOL genotype on the expression of mitochondrial-encoded OXPHOS genes as well as associated, but independently transcribed, nuclear-encoded OXPHOS genes in hybrids. Because of the relative simplicity of the mitochondrial transcription machinery, interpopulation hybrids can be generated that are homozygous for either the maternal mtRPOL genotype (matched mtRPOL-mtDNA genotype) or paternal mtRPOL genotype (mismatched mtRPOL-mtDNA genotype). I hypothesize that hybrids with matched mtRPOL-mtDNA combinations will have OXPHOS transcriptional profiles similar to parental animals, while transcriptional profiles of mismatched mtRPOL-mtDNA genotype hybrids will be altered, particularly for the expression of mitochondrial genes. Using this approach, I can evaluate the level of coadaptation in the mitochondrial transcriptional network within independent lineages and determine how it is affected by hybridization.

Materials and Methods

Generation of recombinant hybrid inbred lines

Interpopulation hybrids were generated for six pairwise crosses beginning with three populations of *T. californicus*: Santa Cruz, California, USA (SCN: 36°57'N, 123°03'W, collected April 2006), Abalone Cove, Palos Verdes, California, USA (AB: 33°44'N, 118°22'W, collected May 2006), and San Diego, California, USA (SD: 32°45'N, 117°15'N 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 liter filtered seawater. Animals were transferred to fresh dishes with each generation.

T. californicus females mate only once and are typically clasped by mature males until reproductively mature. A fine needle was used to separate clasped males and virgin females and the animals were then transferred to appropriate culture dishes to allow repairing and mating with individuals from different populations. Beginning with the three populations listed above (AB, SCN, and SD), all six possible pairwise crosses were generated. For the F_1, F_2, and F_3 hybrid generations, clasped hybrid pairs were removed from culture, separated, and crossed with individuals from a replicate hybrid culture to eliminate the possibility of inbreeding prior to the F_4 generation.

Clasped pairs of F_4 adults were isolated in culture to initiate recombinant hybrid inbred lines. Following the appearance of F_5 juveniles (copepodids), F_4 parental animals were removed from culture for subsequent genotyping of mtRPOL. Only lines determined to be homozygous for mtRPOL were maintained as recombinant hybrid

inbred lines. Lines were inbred by full-sib mating for three fully discrete generations. Offspring of the fourth inbred generation were used to measure OXPHOS transcriptional profiles. Inbred parental lines were generated in parallel as controls from single pairs of population stocks and were inbred for an identical number of generations.

Genotyping of mtRPOL

F_4 hybrid adults used to initiate recombinant hybrid inbred lines were removed from culture and genotyped for mtRPOL using the protocol of Ellison and Burton (2006). Briefly, DNA was prepared by digesting single copepods with 25 µl proteinase-K cell-lysis buffer at 65°C for 1 h followed by 85°C for 15 min. 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). Primer sequences are in Table 4.1. Amplification products were digested overnight with Hinf1 or Msp1 restriction enzymes, respectively (New England Biolabs, Cambridge, MA), and scored on 2% agarose gels following electrophoresis.

Isolation of RNA and generation of cDNA

Animals were subjected to one of two treatments prior to isolation of nucleic acids. A control treatment consisted of transferring animals to 100% seawater at 20°C for 30 minutes prior to initiation of RNA and DNA isolation procedure. In a stress treatment, animals were transferred to a hypoosmotic environment (50% seawater) at 20°C for thirty minutes prior to the isolation of RNA. The nature and duration of this stress treatment were determined empirically with pure strain individuals.

Animals were pooled in groups of five individuals, with all animals within a sample being drawn from a single clutch within a recombinant hybrid inbred line. RNA was isolated using TRI Reagent (Sigma, Saint Louis MO) according to manufacturers specifications. Tissues were disrupted by bead-beating on a Mini-Beadbeater (BioSpec Products, Bartlesville, OK) with zirconia/silica beads for 20 seconds at 4.5 m • sec^{-1}. cDNA synthesis was completed using gene-specific primers (Table 4.1) and the Stratascript Reverse Transcription kit (Stratagene, La Jolla, CA), according to manufacturers specifications, using 30 µl reaction volumes. For each sample, cDNA was generated for alpha tubulin (ATU), and either cytochrome oxidase I (COI) and cytochrome oxidase Va (COVa), cytochrome B (CytB) and cytochrome C (CytC), or ATPase 6 (ATP6) and ATPase c (ATPc). Once first strand cDNA synthesis was complete, samples were stored at –20°C until subsequent quantification assays.

qPCR assays

All qPCR assays were completed on a Stratagene Mx3000P (Stratagene, La Jolla, CA) using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Standards were generated using purified PCR products from parental cDNA samples quantified using a Lambda 35 UV/VIS spectrophotometer (Perkin Elmer, Waltham, MA). Approximate copy number was inferred using OligoCalc (Kibbe 2007) and standards for each target gene were diluted to include concentrations of 10^{11} copies • μl^{-1}, 10^{10} copies • μl^{-1}, 10^{9} copies • μl^{-1}, 10^{8} copies • μl^{-1}, and 10^{7} copies • μl^{-1}.

qPCR was performed in 20 µl reaction volumes containing 8 µl cDNA, 0.25 µg/ml each forward and reverse primer, and 10 µl Power SYBR Green PCR Master Mix

(Applied Biosystems, Foster City, CA). Reaction conditions included a 2 minute denaturing step at 95°C, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 52°C, and 60 seconds at 72°C. Denaturing curves were generated at the end of each series of assays to verify the specificity of the reaction.

Statistical analysis

Quantitative PCR data were normalized to a standard curve. Data for target genes were then normalized to the expression levels of α-tubulin. All statistical analysis used these measures of cDNA abundance corrected for overall transcriptional state (housekeeping gene, α-tubulin). Statistical analysis was performed using SPSS Graduate Pack 11.0 Software (SPSS, Chicago, IL). For crosses involving the AB population (ABxSD, ABxSCN, and the respective reciprocal crosses), data on CytB, CytC, ATPc, and ATP6 were included in the analysis. For the crosses involving the SD and SCN populations, more animals were available for analysis and data on these four loci plus COI and COVa were included.

Data were initially classified using a multivariate discriminant function analysis, including each cross, all six genotypic classes (maternal parent, paternal parent, matched and mismatched mtRPOL-mtDNA genotypes for each cross) for both the control and salinity stress treatments for a total of 72 data categories. Animals from the same hybrid inbred line were treated as identical source material for all analyses. Functions with eigenvalues greater than one were extracted from the data and Wilk's Lambda was used to test (1) equality of group means and (2) power of the primary functions. A structure matrix was calculated for each of the significant discriminant functions to weight the

correlation of each of the variables (the genes assayed) with the principal discriminant functions. One-way ANOVA with a Bonferroni post-hoc multiple comparisons correction ($\alpha = 0.05$) was applied to the significant functions to distinguish the classes of data. Identical one-way ANOVA analyses were used to evaluate stress-treatment data independently of the discriminant function analysis. Sample sizes for each class of data are listed in Table 4.2.

Results

Effect of salinity stress on OXPHOS transcriptional profiles

Previous work indicated that hypoosmotic stress results in a sharp increase in oxygen consumption in *T. californicus* (Goolish and Burton 1989). To evaluate the effect of hypoosmotic stress on OXPHOS transcriptional regulation, we employed a discriminate function analysis incorporating gene expression data for all nuclear and mitochondrial genes studied, for each cross and reciprocal cross, with all mtRPOL-mtDNA genotypic combinations. The parameters of the primary discriminate functions within each subset of data describe the transcriptional profile, based on a set of four or six genes, depending on the cross, for that subset. Results are summarized in Figure 4.1 and Table 4.3 and Table 4.4. No significant transcriptional profile differences were found among genotypes within the control treatment. However, strong differences were found between the control and stress treatments and more complex differences were uncovered among genotypes within the stress treatment (Table 4.4). The primary discriminate function was weighted most strongly with mitochondrial-encoded genes, suggesting that expression of mitochondrial genes contributed more to differences among genotypic classes than did expression of nuclear genes (Table 4.3).

For hybrids bearing either the AB or SCN mtDNA genotypes, control and stress treatment transcriptional profiles of matched mtRPOL-mtDNA hybrids were similar to those of parental controls on both primary discriminant function axes. Mismatched mtRPOL-mtDNA hybrids were significantly different than either parental controls or matched mtRPOL-mtDNA hybrids; transcriptional profiles of mismatched hybrids subjected to hypoosmotic stress were more similar to the control treatment groups than

to the other genotypes subjected to stress (Figure 4.1 and Table 4.4). Hybrids with the SD mtDNA genotype showed a strikingly different pattern in which matched mtRPOL-mtDNA hybrids were significantly different from both parental controls and mismatched mtRPOL-mtDNA hybrids and more similar to the control treatment groups (Figure 4.1 and Table 4.4).

Effect of mtRPOL genotype on OXPHOS transcriptional profiles

The effect of mtRPOL genotype on transcriptional profile was apparent only under hypoosmotic stress conditions; here, we focus only on these stress treatment data. Gene expression for each cross and each gene assayed is presented in Figure 4.2. Mitochondrial gene transcript levels were typically two-fold higher than associated OXPHOS nuclear-encoded genes and expression was much more variable across genotypes for mitochondrial genes. ANOVA results show that five of six crosses had significant differences between genotypic groups for all mitochondrial genes studied, whereas no nuclear gene in any cross had significant expression differences between genotypic classes (Table 4.5). This suggests that the expression of mitochondrial, rather than nuclear, genes is the primary cause of transcriptional profile differentiation based on mtRPOL-mtDNA genotype combinations in hybrids under hypoosmotic stress.

In hybrids bearing either AB or SCN mtDNA, expression of mitochondrial genes under hypoosmotic stress in mismatched mtRPOL-mtDNA hybrids was lower than that of either parental controls or matched mtRPOL-mtDNA hybrids. This effect was significant in three of the four crosses; the cross of SCN female x AB male exhibited the same pattern, though the effect was not significant (Figure 4.2, Table 4.1, and Table 4.6).

No parallel effect was observed for nuclear gene expression. Crosses with SD mtDNA followed a different pattern in which matched mtRPOL-mtDNA hybrids had significantly lower mitochondrial gene expression than either parental or mismatched mtRPOL-mtDNA hybrids. No effect of genotype on expression was observed for nuclear-encoded genes (Figure 4.2, Table 4.5, and Table 4.6). These results indicate that mismatched mtRPOL-mtDNA combinations in hybrids with either AB or SCN mtDNA, and matched mtRPOL-mtDNA combinations for hybrids with SD mtDNA, have a diminished mitochondrial regulatory stress response relative to parental animals.

Viability effects of mtRPOL genotype

In the course of establishing inbred hybrid lines of known mtRPOL genotype, a total of 1,104 F_4 *T. californicus* hybrid adults reared under control conditions were genotyped for mtRPOL. Since lines were initiated as interpopulation hybrids, all F_1 individuals were heterozygotes and the neutral expected frequencies for each of the two mtRPOL alleles in each cross would remain at 50%. Changes in allelic frequencies in generations 2-4 and deviations from Hardy Weinberg expected genotypic proportions provide tests for viability effects. Results are presented in Table 4.7.

Viability effects showed two general patterns: in hybrids with either the AB or the SCN mtDNA genotype, matched mtRPOL-mtDNA was favored, whereas the SD mtRPOL was never favored in hybrids, even with the matched SD mtDNA genotype. The effect was significant in all crosses except for AB female × SCN male. In this case, the direction of the viability effect was the same (favoring the matched genotype), but

was not significant. In each cross, animals assayed for mtRPOL genotype did not significantly deviate from Hardy-Weinberg equilibrium.

Discussion

Enzyme complexes participating in the OXPHOS pathway consist of subunits encoded in both nuclear and mitochondrial genomes. Coordinated gene expression is therefore integral to efficient regulation of the OXPHOS pathway and is complicated by the existence of unique nuclear and mitochondrial transcriptional systems (Taylor and Turnbull 2007). Though regulatory networks of nuclear and mitochondrial OXPHOS genes may not be fully independent, mtRPOL is involved only in the transcription of mitochondrial genes and therefore is not expected to directly impact the expression of nuclear genes. Here, we have found that in interpopulation hybrids of *T. californicus*, mtRPOL genotype has a profound impact on the expression of mitochondrial genes, but no concomitant effect on associated nuclear genes.

The transcriptional profile of OXPHOS genes in interpopulation hybrids of *T. californicus* depends on mtRPOL genotype. Hybrids generated from the AB and SCN populations with matched mtRPOL-mtDNA genotypes have transcriptional profiles similar to parental controls when under hypoosmotic stress, whereas hybrids with mismatched mtRPOL-mtDNA combinations are significantly different. This observation is principally driven by reduced transcript levels of mitochondrial genes in mismatched hybrids; expression of nuclear genes was, as expected, largely unaffected by mtRPOL genotype. The transcriptional profiles of hybrids with mismatched genotypes under stress were further found to be more similar to the control treatment groups than the hypoosmotic stress treatment parental controls, suggesting that mismatched hybrids have a reduced capacity to respond to salinity stress. In sum, these data suggest that coadaptation of mitochondrial transcriptional network components within populations of

T. californicus may be disrupted by hybridization, thereby impairing the regulatory stress response.

In contrast to the above results, hybrids with the SD mtRPOL homozygous genotype, regardless of mtDNA haplotype, were found to have significantly different transcriptional profiles than parental genotypes when under hypoosmotic stress. Hybrids bearing the SD mtRPOL genotype had transcriptional profiles more similar to the unstressed control treatment. Again, this change in transcriptional profile was a result of altered regulation of mitochondrial genes and little change was observed in nuclear gene expression. It therefore appears that the SD mtRPOL genotype has a diminished salinity stress response compared to either the AB or SCN mtRPOL genotypes. Viability effects selecting against the SD mtRPOL genotype in hybrids suggest that the observed regulatory phenotype may be visible to selection pressures and deleterious in hybrids.

Role of transcription factors

The mitochondrial transcription machinery is highly simplified, consisting of only a single-subunit RNA polymerase (mtRPOL) and two transcription factors (Masters et al. 1987; Tiranti et al. 1997). Unlike nuclear transcription systems, mtRPOL itself is responsible for binding strength and specificity to the mitochondrial promoter region and the interaction between mtRPOL and the mitochondrial promoter has been shown to be highly species specific (Gaspari et al. 2004; Matsunaga and Jaehning 2004). This suggests that coadaptation of mtRPOL and the mitochondrial promoter may evolve within isolated lineages. In crosses between the AB and SCN populations of *T. californicus*, matched mtRPOL-mtDNA combinations are favored over mismatched

combinations in hybrids. This may reflect coadaptation between mtRPOL and the mitochondrial promoter evolving within these populations to improve binding specificity and strength independent of transcription factors.

Though only two transcription factors, TFAM and either TFB1M or TFB2M, are generally involved in mitochondrial transcription (Masters et al. 1987; Tiranti et al. 1997), a number of accessory proteins also appear to be involved in mitochondrial regulatory processes (Falkenberg et al 2007; Park et al 2007). Crosses involving the SD population repeatedly revealed poor function of SD-derived mtRPOL. Remarkably, hybrids with a matched SD mtRPOL-mtDNA genotype were unable to mirror the SD parental transcriptional profile, clearly indicating that genes other than mtRPOL are involved in maintaining the efficacy of the SD mitochondrial transcriptional system in parental lineages. TFB1M has been shown to be in tight linkage with mtRPOL in *T. californicus* (Flowers 2005) and is therefore unlikely to contribute to the SD mtRPOL dysfunction in hybrids described herein. The role of TFAM and other regulatory accessory proteins in maintaining this regulatory network, however, remains open to investigation.

Evolution of the mitochondrial transcriptional network in T. californicus

The mechanisms by which hybrid incompatibilities arise are frequently understood in terms of the Dobzhansky-Muller model (Dobzhansky 1936; Muller 1942). This model suggests that neutral or nearly neutral mutations accumulating within lineages become deleterious only when placed on a different genetic background, thus permitting the evolution of deleterious incompatibility loci between lineages without

strong selection acting against any combination of the same loci within lineages. Landry et al (2007) argue that the complexity of regulatory networks creates "degeneracy" analogous to the redundant nature of the genetic code. In this way, neutral variation may segregate within lineages and eventually diverge from sister groups. However, the reduced complexity of the mitochondrial transcriptional regulatory network compared to nuclear regulatory networks may reduce the applicability of such a model with regard to mitochondrial transcription (Johnson and Porter 2000). An alternative model describing the generation of hybrid incompatibilities was proposed by Kimura (1985). Under this model of compensatory evolution, deleterious mutations are balanced within lineages by secondary mutations that yield the combined epistatic network nearly neutral; dissolution of these epistatic interactions and their compensatory mechanisms in hybrids subsequently exposes the deleterious mutations to selection.

A Dobzhansky-Muller process may explain the pattern of mitochondrial regulatory dysfunction in crosses between the AB and SCN populations of *T. californicus*, wherein each population appears to have coadapted mtRPOL-mtDNA genotypes. However, this same model is unlikely to explain the pattern observed in crosses involving the SD population. It is clear that the SD mtRPOL genotype is deleterious when placed in a hybrid genomic background. A combination of SD mtRPOL and SD mtDNA in hybrids is not sufficient to reconstitute the SD parental mitochondrial transcriptional profile, therefore one or more deleterious mutations in the SD mitochondrial transcription machinery must be maintained by an unlinked compensatory locus. This could involve a mitochondrial transcription factor (such as

TFAM) or accessory protein, though this study lacks the resolution to specifically identify the relevant compensatory locus or loci.

The evolution of cis-trans regulatory interactions, and their modification in hybrids, has been surveyed extensively (Wittkopp et al. 2004, 2008; Landry et al. 2005), but little is known about individual cis-trans interactions between promoters and RNA polymerases, transcription factors, or accessory proteins in these cases. Here, we evaluate the effect of hybridization specifically on the mitochondrial regulatory system and find evidence (1) that mtRPOL genotype has a large impact on mitochondrial transcription in hybrids and (2) that mitochondrial regulatory dysfunction may result in decreased responsiveness to stress conditions. Such an effect may have broadly pleiotropic effects on hybrid fitness, including delayed development and reduced fecundity and survivorship. We conclude that compensatory epistatic mutations in the mitochondrial transcriptional regulatory system within populations of *T. californicus* are disrupted by interpopulation hybridization. This may contribute to a reduced stress response capacity and, consequently, reduced fitness in hybrids.

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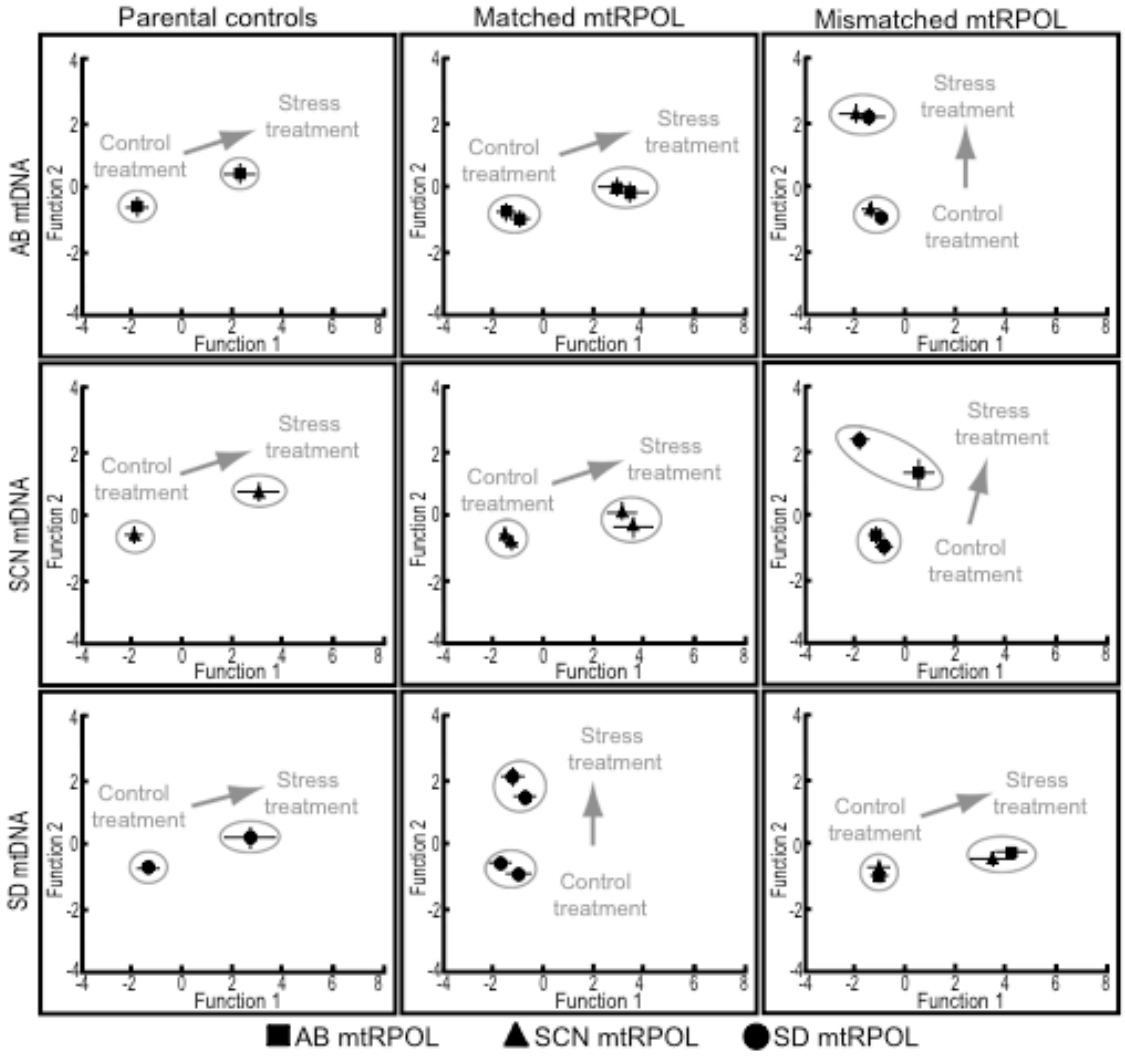

Figure 4.1: Salinity stress affects OXPHOS transcriptional profile in interpopulation hybrids. Discriminant function analysis results showing effects of salinity stress on transcriptional profile in hybrids and parental animals. Data are grouped by mtDNA genotype and parental, matched, or mismatched hybrid mtRPOL genotypes. Mean and standard errors shown for each treatment and mtRPOL genotype class.

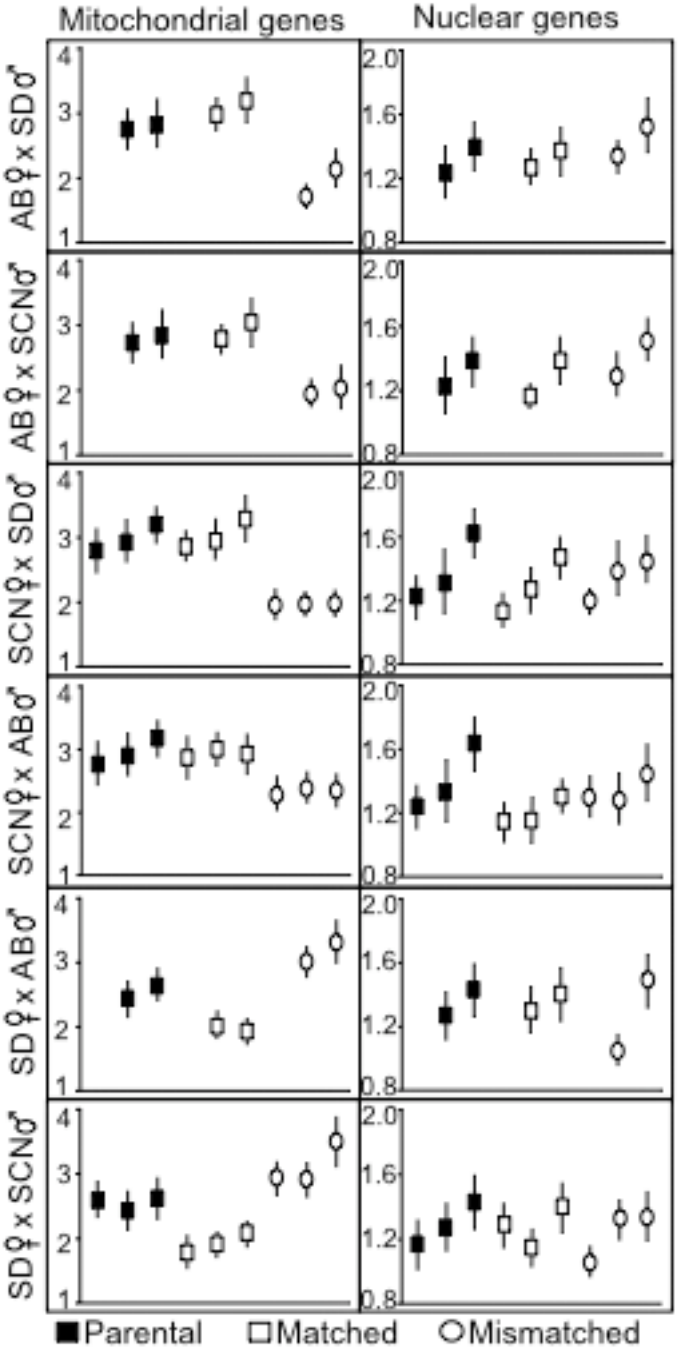

Figure 4.2: Transcript levels among mtDNA-mtRPOL genotype classes for mitochondrial and nuclear genes. Mean gene expression measures in hypoosmotic stress treatment only shown for each gene and cross with respective standard error (all values $x10^5$). Mitochondrial gene expression values (left column) are presented, left to right, as COI, CytB, and ATP6; nuclear gene expression values (right column) are presented, left to right, as COVa, CytC, and ATPc.

Table 4.1: Primer sequences for mtRPOL genotyping assays and qPCR assays.

Gene	Primer Name	5'-3' sequence
mtRPOL genotyping		
mtRPOL	MTRP-gtype.1F	CGTGTTTATCCCATCCCT
	MTRP-gtype.1R	CGAAGCTATACAAGCG
	MTRP-Msp1.F	GCGTATGCAATATTGCCGAGG
	MTRP-Msp1.R	TCTTTGGATAGCTCCATTCAAG
qPCR		
COVa	COVa.34F	ACCGACGCCGAGTTTGAT
	COVa.345R	AATTTCCTGGAGCATGTAGG
COI	COI.SD.113F	CGTTTAGAGCTTGGACAGTG
	COI.SD.381R	AGGATACACAGTTCACCCAG
	COI.SCN.118F	CAGTGTGGTAGCTTTTTAGG
	COI.SCN.444R	CCAAATGAAGAGAGAAGATAG
CytC	CytC.160F	CACGGACATCATCGAGTATG
	CytC.427R	CGAGGCTCTAGCGTTCATGT
CytB	CytB.SD.188F	TTTCGTGCTCTCCACGCTAA
	CytB.SD.458R	AGAAGCCCCCTCACAATCAA
	CytB.AB.537F	TGGGCTTCATTTTGTGTTGC
	CytB.AB.809R	GGCTGAATATGGTGGGGTGT
	CytB.SCN.415F	TCCTTCTGAGGGGCAACAGT
	CytB.SCN.752R	CCCAGAAGCCACGGATAAAA
ATPc	ATPc. 148F	CCAAGTTCATCGGAGCTGGT
	ATPc.345R	GGAGCAAAAAGGCCATCATC
ATP6	ATP6.SD.306F	TGTATCGGCAGCCTTTATGG
	ATP6.SD.623R	CCTTGGTCTCGGACTCCCTA
	ATP6.AB.310F	TAAGCAGCCCTTGTGGGTTT
	ATP6.AB.567R	TGAATGATGCACACCGCTAA
	ATP6.SCN.255F	TCACGCTAAGGCTGGCACTA
	ATP6.SCN.526R	CAGAACACCCAAACCTGCAA
α-tubulin	ATU.79F	AGATCACCAACGCCTGCT
	ATU.415R	ACACGGCACGGGGAACTT

Table 4.2: Sample sizes for each category of data.

COVa

		Normal treatment		Stress treatment	
		Match	Mismatch	Match	Mismatch
Parental	SD	12	—	12	—
Population	AB	12	—	12	—
Controls	SCN	12	—	12	—
	SD × AB	—	—	—	—
	AB × SD	—	—	—	—
Hybrid	SD × SCN	10	10	10	11
Lineages	SCN × SD	11	11	11	11
	AB × SCN	—	—	—	—
	SCN × AB	9	10	10	10

COI

		Normal treatment		Stress treatment	
		Match	Mismatch	Match	Mismatch
Parental	SD	12	—	12	—
Population	AB	12	—	12	—
Controls	SCN	12	—	12	—
	SD × AB	—	—	—	—
	AB × SD	—	—	—	—
Hybrid	SD × SCN	10	10	10	11
Lineages	SCN × SD	11	11	11	11
	AB × SCN	—	—	—	—
	SCN × AB	9	10	10	10

CytC

		Normal treatment		Stress treatment	
		Match	Mismatch	Match	Mismatch
Parental	SD	12	—	12	—
Population	AB	12	—	12	—
Controls	SCN	12	—	12	—
	SD × AB	10	10	10	10
	AB × SD	10	10	11	11
Hybrid	SD × SCN	11	11	11	11
Lineages	SCN × SD	11	11	12	12
	AB × SCN	10	9	11	11
	SCN × AB	12	12	12	12

Table 4.2: Sample sizes for each category of data, continued.

CytB

		Normal treatment		Stress treatment	
		Match	Mismatch	Match	Mismatch
Parental Population Controls	SD	12	—	12	—
	AB	12	—	12	—
	SCN	12	—	12	—
Hybrid Lineages	SD × AB	10	10	10	10
	AB × SD	10	10	11	11
	SD × SCN	11	11	11	11
	SCN × SD	11	11	12	12
	AB × SCN	10	10	11	11
	SCN × AB	12	12	12	12

ATPc

		Normal treatment		Stress treatment	
		Match	Mismatch	Match	Mismatch
Parental Population Controls	SD	12	—	12	—
	AB	12	—	12	—
	SCN	12	—	12	—
Hybrid Lineages	SD × AB	10	10	9	10
	AB × SD	10	10	10	11
	SD × SCN	12	12	12	12
	SCN × SD	12	12	12	12
	AB × SCN	12	12	12	12
	SCN × AB	12	12	12	12

ATP6

		Normal treatment		Stress treatment	
		Match	Mismatch	Match	Mismatch
Parental Population Controls	SD	12	—	12	—
	AB	12	—	12	—
	SCN	12	—	12	—
Hybrid Lineages	SD × AB	10	10	9	10
	AB × SD	10	10	10	11
	SD × SCN	12	12	12	12
	SCN × SD	12	12	12	12
	AB × SCN	12	12	12	12
	SCN × AB	12	12	12	12

Table 4.3: Structure matrix for discriminant function analysis with eigenvalues and percent of total variance explained for significant discriminant functions recovered. Mitochondrial genes are more strongly weighted for the primary discriminant function, while all genes are nearly equally weighted for the secondary discriminant function.

		DF 1	DF 2
MtDNA	CytB	0.515	0.482
	ATP6	0.657	0.475
Nuc DNA	CytC	0.198	0.585
	ATPc	0.176	0.653
Eigenvalues		4.589	1.291
Percent variance		76%	22%

Table 4.4: ANOVA of primary discriminant functions. No significant differences were found among control treatment genotype classes and all control treatment genotype classes were significantly different than all stress treatment genotype classes (data not shown). More complex results resulted from comparisons between genotypes within hypoosmotic stress treatment. Results for discriminant function 1 are shown above the diagonal; results for discriminant function 2 are shown below the diagonal. Data are grouped by mtDNA genotype. ANOVA with Bonferroni post-hoc comparisons (α=0.05)

AB mtDNA

		Matched		**Mismatched**	
	AB	ABxSD	ABxSCN	ABxSD	ABxSCN
AB	---	0.3984	1.0000	<0.0001	<0.0001
ABxSD	0.1670	---	0.1004	<0.0001	0.0068
ABxSCN	1.0000	0.2332	---	<0.0001	<0.0001
SDxAB	0.0002	<0.0001	<0.0001	---	1.0000
SCNxAB	0.0004	<0.0001	<0.0001	1.0000	---

SCN mtDNA

		Matched		Mismatched	
	SCN	SCNxSD	SCNxAB	SCNxSD	SCNxAB
SCN	---	1.0000	1.0000	<0.0001	0.0012
SCNxSD	1.0000	---	1.0000	<0.0001	0.0005
SCNxAB	0.6734	1.0000	---	<0.0001	<0.0001
SDxSCN	0.0489	0.0024	0.0001	---	0.0071
ABxSCN	1.0000	0.4030	0.0439	0.7301	---

SD mtDNA

		Matched		Mismatched	
	SD	SDxAB	SDxSCN	SDxAB	SDxSCN
SD	---	<0.0001	<0.0001	0.0009	0.0227
SDxAB	0.7900	---	1.0000	<0.0001	<0.0001
SDxSCN	1.0000	1.0000	---	<0.0001	<0.0001
ABxSD	0.3383	0.0046	0.0390	---	1.0000
SCNxSD	0.1051	0.0010	0.0151	1.0000	---

Table 4.5: Significant differences between genotypic classes under hypoosmotic stress for mitochondrial, but not nuclear, gene expression. ANOVA of gene expression data partitioned by cross and gene for hypoosmotic stress treatment only. Crosses shown as (female parent population) x (male parent population). All comparisons comprise maternal parent, matched and mismatched mtDNA-mtRPOL genotype hybrids within each cross (* denotes significant result at α=0.05).

		ABxSD	**ABxSCN**	**SCNxSD**	**SCNxAB**	**SDxAB**	**SDxSCN**
	COI	n.d.	n.d.	0.0248 *	0.2523	n.d.	0.0273 *
mtDNA	CytB	0.0051 *	0.0133 *	0.0487 *	0.1124	0.0425 *	0.0433 *
	ATP6	0.0366 *	0.0311 *	0.0071 *	0.0978	0.0215 *	0.0118 *
	COVa	n.d.	n.d.	0.8163	0.6995	n.d.	0.5231
gDNA	CytC	0.8575	0.8346	0.4598	0.7316	0.4330	0.6404
	ATPc	0.7507	0.7638	0.8817	0.4763	0.9340	0.9450

Table 4.6: ANOVA within cross, genes, and treatments ($\alpha = 0.003$).

Cross	Gene	Treatment	P	Comparison	P	Treatment	Gene
SD × AB	COVa	Normal	—	Parent vs Match	—	Normal	COI
			—	Parent vs Mismatch	—		
			—	Match vs Mismatch	—		
		Stress	—	Parent vs Match	—	Stress	
			—	Parent vs Mismatch	—		
			—	Match vs Mismatch	—		
	CytC	Normal	0.2602	Parent vs Match	0.2256	Normal	CytB
			1	Parent vs Mismatch	1		
			1	Match vs Mismatch	0.0342		
		Stress	<0.0001	Parent vs Match	0.0044	Stress	
			0.5055	Parent vs Mismatch	0.0118		
			0.0001	Match vs Mismatch	<0.0001		
	ATPc	Normal	0.1006	Parent vs Match	0.8852	Normal	ATP6
			0.0481	Parent vs Mismatch	0.0667		
			1	Match vs Mismatch	0.0071		
		Stress	0.0139	Parent vs Match	0.0006	Stress	
			0.0002	Parent vs Mismatch	0.3346		
			0.4993	Match vs Mismatch	<0.0001		

Cross	Gene	Treatment	P	Comparison	P	Treatment	Gene
AB × SD	COVa	Normal	—	Parent vs Match	—	Normal	COI
			—	Parent vs Mismatch	—		
			—	Match vs Mismatch	—		
		Stress	—	Parent vs Match	—	Stress	
			—	Parent vs Mismatch	—		
			—	Match vs Mismatch	—		
	CytC	Normal	0.0019	Parent vs Match	0.4315	Normal	CytB
			0.1597	Parent vs Mismatch	0.5671		
			0.2832	Match vs Mismatch	1		
		Stress	<0.0001	Parent vs Match	0.0588	Stress	
			<0.0001	Parent vs Mismatch	<0.0001		
			1	Match vs Mismatch	<0.0001		
	ATPc	Normal	0.0015	Parent vs Match	0.0001	Normal	ATP6
			0.0227	Parent vs Mismatch	0.0009		
			0.9779	Match vs Mismatch	1		
		Stress	0.0089	Parent vs Match	1	Stress	
			<0.0001	Parent vs Mismatch	<0.0001		
			0.0177	Match vs Mismatch	<0.0001		

Table 4.6: ANOVA within cross, genes, and treatments ($\alpha = 0.003$), continued.

Cross	Gene	Treatment	P	Comparison	P	Treatment	Gene
SD × SCN	COVa	Normal	<0.0001	Parent vs Match	1	Normal	COI
			<0.0001	Parent vs Mismatch	0.6483		
			1	Match vs Mismatch	0.2779		
		Stress	<0.0001	Parent vs Match	<0.0001	Stress	
			<0.0001	Parent vs Mismatch	1		
			0.1453	Match vs Mismatch	<0.0001		
	CytC	Normal	0.0088	Parent vs Match	1	Normal	CytB
			0.0106	Parent vs Mismatch	1		
			1	Match vs Mismatch	1		
		Stress	<0.0001	Parent vs Match	0.0005	Stress	
			<0.0001	Parent vs Mismatch	1		
			1	Match vs Mismatch	0.0018		
	ATPc	Normal	0.0003	Parent vs Match	0.3309	Normal	ATP6
			<0.0001	Parent vs Mismatch	1		
			0.0262	Match vs Mismatch	0.2281		
		Stress	<0.0001	Parent vs Match	0.0008	Stress	
			<0.0001	Parent vs Mismatch	1		
			0.5807	Match vs Mismatch	0.0001		

Cross	Gene	Treatment	P	Comparison	P	Treatment	Gene
SCN × SD	COVa	Normal	0.0142	Parent vs Match	0.4689	Normal	COI
			0.0014	Parent vs Mismatch	0.2523		
			1	Match vs Mismatch	1		
		Stress	0.0312	Parent vs Match	0.0496	Stress	
			0.0005	Parent vs Mismatch	0.0003		
			0.4195	Match vs Mismatch	<0.0001		
	CytC	Normal	<0.0001	Parent vs Match	1	Normal	CytB
			0.0004	Parent vs Mismatch	1		
			1	Match vs Mismatch	1		
		Stress	<0.0001	Parent vs Match	1	Stress	
			<0.0001	Parent vs Mismatch	<0.0001		
			<0.0001	Match vs Mismatch	<0.0001		
	ATPc	Normal	<0.0001	Parent vs Match	1	Normal	ATP6
			0.0086	Parent vs Mismatch	1		
			0.0046	Match vs Mismatch	1		
		Stress	<0.0001	Parent vs Match	0.0325	Stress	
			0.002	Parent vs Mismatch	<0.0001		
			0.5425	Match vs Mismatch	0.0018		

Table 4.6: ANOVA within cross, genes, and treatments ($\alpha = 0.003$), continued.

Cross	Gene	Treatment	P	Comparison	P	Treatment	Gene
AB × SCN	COVa	Normal	—	Parent vs Match	—	Normal	COI
			—	Parent vs Mismatch	—		
			—	Match vs Mismatch	—		
		Stress	—	Parent vs Match	—	Stress	
			—	Parent vs Mismatch	—		
			—	Match vs Mismatch	—		
	CytC	Normal	0.2492	Parent vs Match	1	Normal	CytB
			0.0068	Parent vs Mismatch	1		
			0.4425	Match vs Mismatch	1		
		Stress	<0.0001	Parent vs Match	1	Stress	
			<0.0001	Parent vs Mismatch	<0.0001		
			1	Match vs Mismatch	0.0006		
	ATPc	Normal	<0.0001	Parent vs Match	0.1627	Normal	ATP6
			<0.0001	Parent vs Mismatch	<0.0001		
			1	Match vs Mismatch	0.0222		
		Stress	<0.0001	Parent vs Match	1	Stress	
			<0.0001	Parent vs Mismatch	<0.0001		
			1	Match vs Mismatch	<0.0001		

Cross	Gene	Treatment	P	Comparison	P	Treatment	Gene
SCN × AB	COVa	Normal	<0.0001	Parent vs Match	1	Normal	COI
			<0.0001	Parent vs Mismatch	0.1052		
			0.4107	Match vs Mismatch	0.0414		
		Stress	0.0015	Parent vs Match	1	Stress	
			<0.0001	Parent vs Mismatch	0.0493		
			0.1854	Match vs Mismatch	0.0166		
	CytC	Normal	0.0002	Parent vs Match	1	Normal	CytB
			<0.0001	Parent vs Mismatch	0.6124		
			0.3907	Match vs Mismatch	1		
		Stress	<0.0001	Parent vs Match	1	Stress	
			<0.0001	Parent vs Mismatch	0.0644		
			<0.0001	Match vs Mismatch	0.0158		
	ATPc	Normal	0.0001	Parent vs Match	1	Normal	ATP6
			<0.0001	Parent vs Mismatch	1		
			0.8003	Match vs Mismatch	1		
		Stress	0.0601	Parent vs Match	0.0077	Stress	
			0.0113	Parent vs Mismatch	<0.0001		
			1	Match vs Mismatch	0.1216		

Table 4.7: Viability effects of mtRPOL genotype in interpopulation hybrids. Genotypic numbers and chi-square test to Hardy-Weinberg expectations for mtRPOL genotypes in F_4 hybrid adults reared under control conditions. Also shown are the mtRPOL allelic frequencies (matched = same source population as for the mtDNA) and chi-square test for deviation from the neutral expected frequency of 0.5. All crosses are listed as (female parent population) x (male parent population).

	mtRPOL genotypes			**Hardy-Weinberg**		**Allele Frequencies**		
ABxSCN	Match	Heterozygote	Mismatch					
Observed	69	94	50	χ^2 =	2.5909	p_{match} =	0.54	χ^2 = 3.206
Expected	63	106	44	p =	0.1075	$p_{mismatch}$ =	0.46	p = 0.073
ABxSD								
Observed	77	79	32	χ^2 =	1.2549	p_{match} =	0.62	χ^2 = 21.543
Expected	72	89	27	p =	0.2626	$p_{mismatch}$ =	0.38	p = 0
SCNxAB								
Observed	67	115	35	χ^2 =	1.5118	p_{match} =	0.57	χ^2 = 9.428
Expected	71	106	39	p =	0.2188	$p_{mismatch}$ =	0.43	p = 0.002
SCNxSD								
Observed	62	71	17	χ^2 =	0.2435	p_{match} =	0.65	χ^2 = 26.316
Expected	63	68	18	p =	0.6217	$p_{mismatch}$ =	0.35	p = 0
SDxAB								
Observed	22	61	59	χ^2 =	0.8698	p_{match} =	0.37	χ^2 = 19.282
Expected	19	66	56	p =	0.3510	$p_{mismatch}$ =	0.63	p = 0
SDxSCN								
Observed	30	89	75	χ^2 =	0.1781	p_{match} =	0.38	χ^2 = 20.876
Expected	29	92	74	p =	0.6730	$p_{mismatch}$ =	0.62	p = 0

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