DISRUPTION OF MITOCHONDRIAL FUNCTION IN INTERPOPULATION HYBRIDS OF *TIGRIOPUS CALIFORNICUS*

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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. We 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, we measured fitness, mitochondrial function, and ETS enzyme activity in inbred recombinant hybrid lines of *Tigriopus californicus*. We found 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 exist between ETS enzyme activity and mitochondrial function and between mitochondrial function and fitness. We also present evidence that hybrid lines harboring mitochondrial DNA (mtDNA) and mitochondrial RNA polymerase (mtRPOL) 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.

Key words.—Copepod, electron transport system, hybrid breakdown, intergenomic coadaptation, intrinsic post-zygotic isolation, mitochondrial function, mitochondrial RNA polymerase.

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 western coast of North America using allozymes and nuclear and mitochondrial DNA (mtDNA) 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

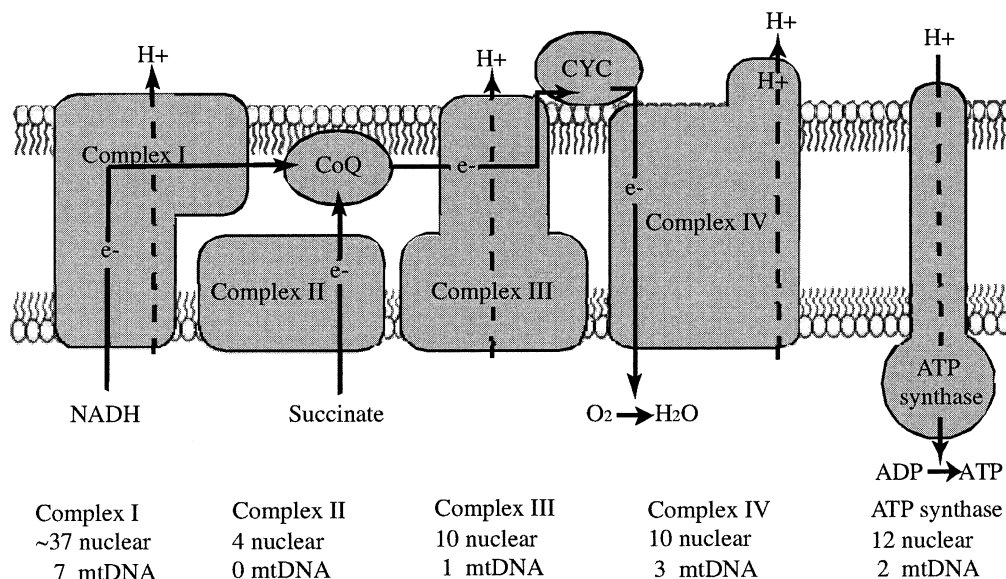| Complex I	Complex II	Complex III	Complex IV	ATP synthase
~37 nuclear	4 nuclear	10 nuclear	10 nuclear	12 nuclear
7 mtDNA	0 mtDNA	1 mtDNA	3 mtDNA	2 mtDNA

FIG. 1. Diagram of electron transport system 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.

(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, whereas complex II is wholly nuclear-encoded (see Fig. 1) (Saraste 1999). Complex II therefore provides an interesting internal control by which to examine the strength of nuclear-mitochondrial interaction in hybrid animals, because activity of this complex is expected to be independent of mitochondrial background, whereas 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. 2002). 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, we 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, we determine the mitochondrial RNA polymerase genotype for each of the inbred recombinant hybrid lines and compare the mitochondrial function of matched versus mismatched genotypes. We 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, California (SCN: 36°57′N,

122°03′W, collected October 2002), Abalone Cove, Palos Verdes Peninsula, California (AB: 33°44′N, 118°22′W, collected May 2003), San Diego, California (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.

Tigriopus 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 1966; 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 × SCN) as well as interpopulation hybrid animals (e.g., SD × SCN). Crosses were performed with four replicates, each containing five males from one population and five 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, although 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 six-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 1-ml 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 × 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 × 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. Of 108 mitochondrial isolations, four 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 quantification 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 $1\times$ 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 h 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 3 h; the 2-h incubation was selected to be certain that 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

J. M. Flowers and R. S. Burton (unpubl. ms.) 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 (unpubl. data). DNA was prepared by digesting single copepods with 25 µl proteinase-K cell-lysis buffer at 65°C for 1 h followed by 100°C for 15 min. Briefly, for crosses of PM × SD, AB × SD, and SCN × SD and their reciprocal crosses, polymerase chain reaction (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 95°C, 1 min at 50°C, and 2 min at 72°C. 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 × SCN and AB × 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 95°C, 30 sec at 54°C, and 1 min at 72°C. These products were digested using Msp1 restriction enzymes and buffers and scored on 2% agarose gels following electrophoresis. PM × 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 χ^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 1. Hybrid lines were observed to have a significantly lower hatching number than parental control lines ($P < 0.001$), with a decrease of more than 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 ($P = 0.012$). Furthermore, the standard error for hatching number, survivorship, and metamorphosis increased by approximately 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.

TABLE 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	Complex I	Complex II	Complex III	Complex IV	Citrate synthase
Parental lines mean (SE)	33.125 (1.429)	0.899 (0.011)	0.513 (0.020)	1.459 (0.078)	3.200 (0.067)	8.217 (0.267)	16.017 (0.217)	3.150 (0.050)	4.433 (0.150)
Hybrid lines mean (SE)	19.05 (2.763)	0.851 (0.019)	0.377 (0.046)	0.9857 (0.102)	2.25 (0.133)	8.417 (0.533)	11.000 (0.533)	2.717 (0.083)	3.933 (0.267)
Significance	<0.001	0.039	0.012	0.001	<0.001	0.735	<0.001	<0.001	0.112

A)

Hatching number versus ATP production

p = 0.212
slope = 4.634

Number of nauplii

ATP production (nmol ATP/mg protein/min)

B)

Survivorship versus ATP production

p = 0.037
slope = 0.063

Survivorship fraction

ATP production (nmol/mg protein/min)

C)

Metamorphosis versus ATP production

p = 0.304
slope = 0.127

Metamorphosis fraction

ATP production (nmol ATP/mg protein/min)
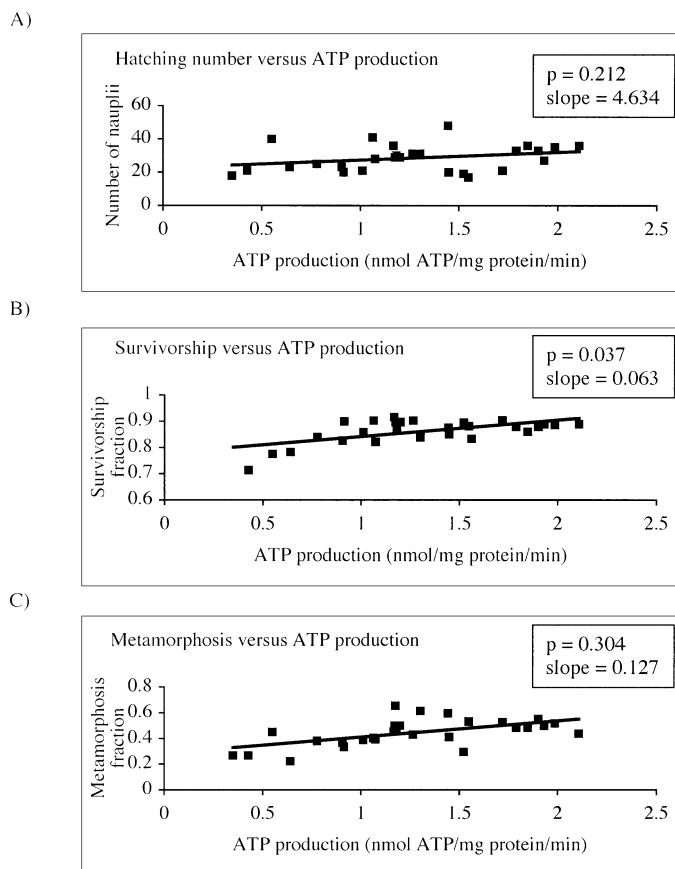
FIG. 2. Correlations between (A) hatching number and rate of ATP production, (B) survivorship and rate of ATP production, and (C) metamorphosis and rate of ATP production. Significance based on Pearson's correlation and slopes calculated based on least-squares regression (regression lines shown on figure).

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 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 ($P = 0.001$). Furthermore, 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 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 1. Significant declines in mean activity were observed in hybrid lines for complex I, complex III, and complex IV, whereas 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%, although 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, although 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 twofold 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.

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 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 was 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 ($\chi^2 = 8.909$, df = 1, $P = 0.003$). Mismatched hybrid lines performed more poorly than matched hybrid lines for measures of hatching number, survivorship, metamorphosis, ATP production, and complex III and complex IV activities, although this effect was only significant for
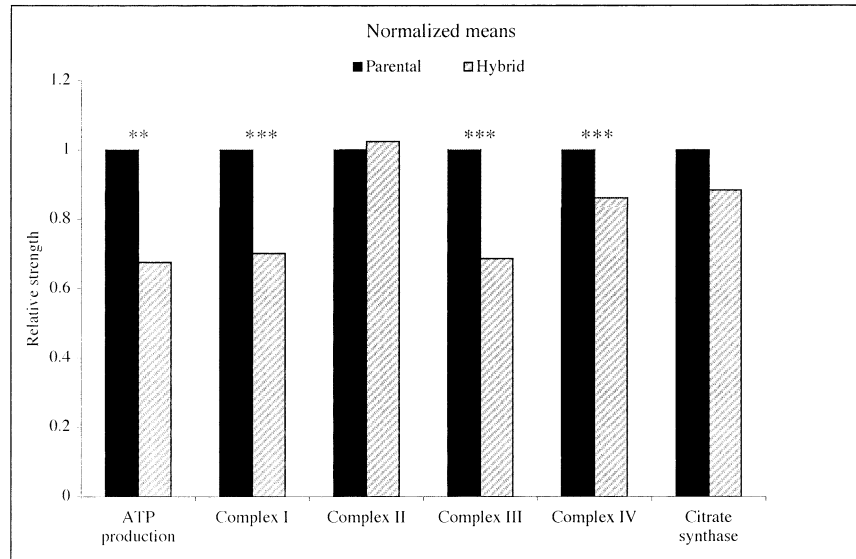
FIG. 3.   Mean values of ATP production rate and activity of complexes I, II, III, and IV, and citrate synthase for inbred parental control lines and inbred recombinant hybrid lines normalized to parental control line values. ** $P < 0.01$, *** $P < 0.0001$.

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, although in no case was this effect significant (Fig. 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 we 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) whether 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*
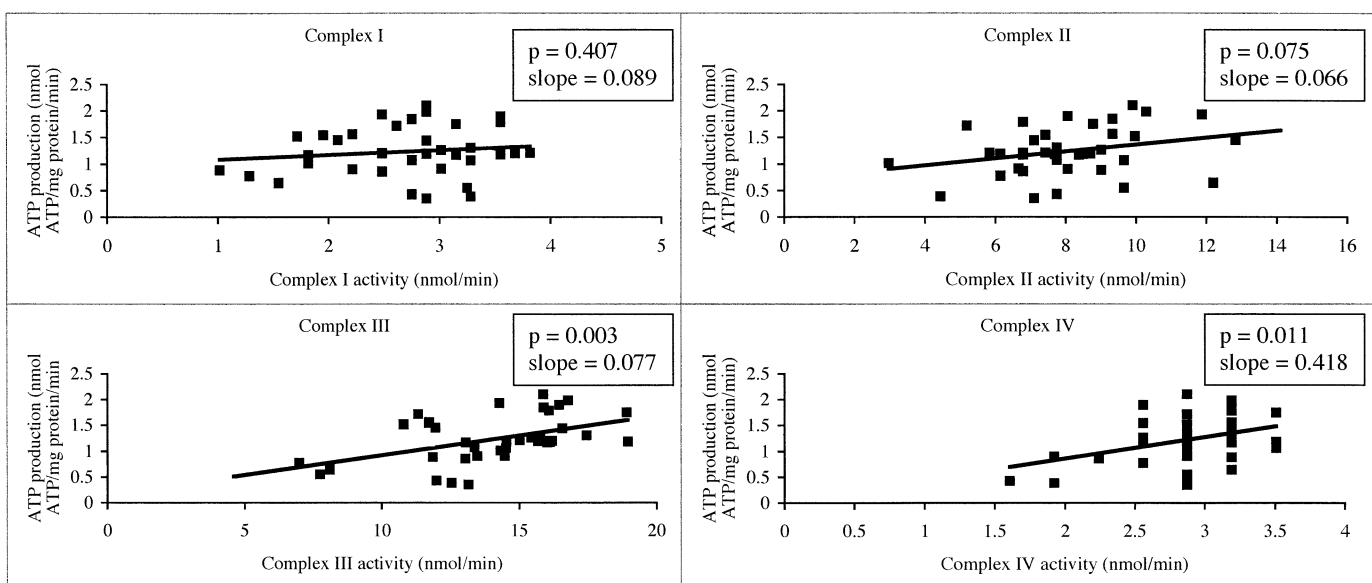

FIG. 4.   Correlations between rate of ATP production and (A) complex I activity, (B) complex II activity, (C) complex III activity, and (D) complex IV activity. Significance based on Pearson's correlation and slopes calculated based on least-squares regression (regression lines shown on figure).
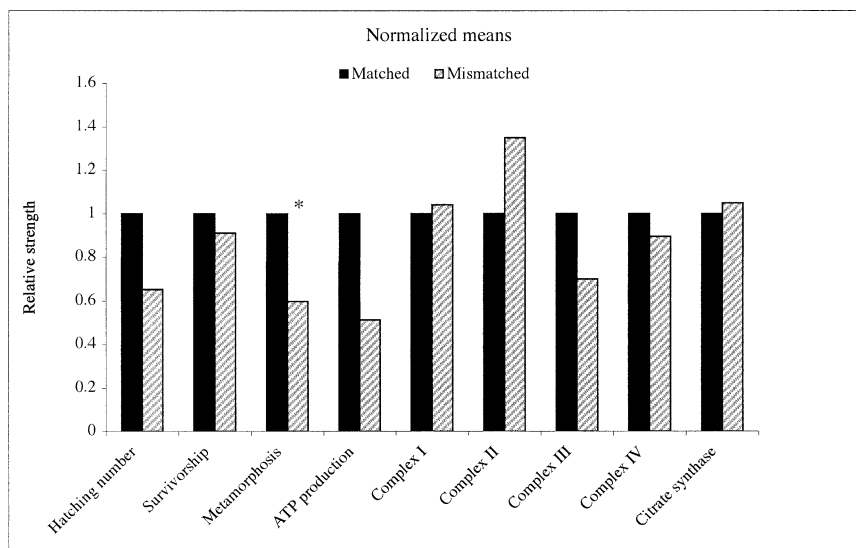
Fig. 5. Mean values of fitness parameters, ATP production rate; activity of complexes I, II, III, and IV; and citrate synthase for inbred recombinant hybrid lines containing mitochondrial RNA polymerase and mitochondrial genotypes derived from the same parental population (matched) and derived from different parental populations (mismatched). Data normalized to mean values for matched lines. * $P < 0.05$.

have shown a high degree of variance among individuals in the 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. We 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 we grouped all hybrid lines together and all control lines together, although 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, we 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 subcellular 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 protein function, we 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, although models of nuclear-mitochondrial interactions provide the only significant fit to the data. (Edmands and Burton 1999). Although 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, unpubl. data). Although 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 nonsynonymous substitution rate at *T. californicus* nuclear versus mitochondrial loci. Although 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, then, is 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, because 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, although 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 we 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 organ-

ismal fitness. We 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. We 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 we 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, although 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 postzygotic isolating barrier for conspecific populations having strong genetic divergence.

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