Functional Divergence of Mitochondria and Coevolution of Genomes: Cool Mitochondria in Hot Lizards

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ABSTRACT

Mitochondria play a key role in the ecology and evolution of species through their influence on aerobic metabolism. Mitochondrial DNA (mtDNA) and nuclear genomes must interact for optimal functioning of oxidative phosphorylation to produce ATP, and breakdown of coadaptation components from each may have important evolutionary consequences for hybridization. Introgression of mitochondria in natural populations through hybridization with unidirectional backcrossing allows the testing of coadaptation of mitochondria to different nuclear backgrounds. We compared the function of mitochondria isolated from two species of *Urosaurus* lizards and hybrid populations. Due to past introgression, hybrids contain the nuclear genome of the "hot-adapted" species (*U. graciosus*) but the mtDNA of the less heat-tolerant species (*U. ornatus*). It was found that the function of the parental forms of mitochondria had significantly diverged with the hot-adapted species. There was significant genotype × genotype × environment interactions for mitochondrial membrane potential and genotype × genotype interactions for ATP production. Membrane potential decreased less at a higher temperature, while ATP production was higher at both temperatures in introgressed mitochondria. Oxygen consumption was lower in *U. graciosus* than in *U. ornatus* parental-type mitochondria, indicating a likely response to living in hotter environments. Respiratory control ratio values, which provide an indication of the functional quality of isolated mitochondria, were lower in introgressed mitochondria than in parental *U. ornatus* types, indicating a negative impact on biological function in introgressed mitochondria.

Keywords: mitochondrial membrane potential, ATP production, introgression, coevolution, mitochondrial DNA (mtDNA), lizard.

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Introduction

Thermal environments can cause strong selection on organisms, and evolutionary responses of metabolic processes can be important for adaptation to different thermal conditions (Weathers 1979; Bozinovic and Rosenmann 1989; Hosken and Withers 1997; Rezende et al. 2004; Swanson and Garland 2009). Mitochondria are responsible for aerobic respiration in cells of metazoans and therefore play an important role in shaping the metabolism and life histories of organisms (see Ballard and Melvin 2010). Mitochondria have likely evolved to adjust rates of oxidative phosphorylation (OXPHOS) to specific environmental conditions of organisms, including their thermal environments (Das 2006).

Mitochondria have reduced genomes separate from the nucleus, and mitochondrial DNA (mtDNA) variation can contribute to processes of aging (Camus et al. 2012), male-specific dysfunctions (Frank and Hurst 1996; Rand et al. 2001; Dowling et al. 2007; Smith et al. 2010), evolution of flight (bats: Shen et al. 2010; insects: Yang et al. 2014), and thermal adaptation (Mishmar et al. 2003; Fangue et al. 2009; Baris et al. 2016; Camus et al. 2017). Mitochondrial variants are also mechanistically linked to numerous diseases in humans (Taylor and Turnbull 2005; Schapira 2006; Schaefer et al. 2008). Experimental crosses that controlled for nuclear background identified functional differences of mitochondria in *Drosophila*, copepods, and killifish (Edmans and Burton 1998; Willett and Burton 2001, 2004; Sackton et al. 2003; Ellison and Burton 2006, 2008*a*, 2008*b*; Fangue et al. 2009; Levin et al. 2014; Hill 2016; Wolff et al. 2016). These experiments indicate that variation of mitochondria found in natural systems can have important evolutionary implications (see Ballard and Pichaud 2014).

While OXPHOS takes place in the mitochondria, only 13 of the hundreds of proteins used in OXPHOS are coded in the mitochondrial genome (see Zhang and Broughton 2013; Ballard and Pichaud 2014; Calvo et al. 2016). The mitochondrial-encoded proteins must interact with their nuclear counterparts involved in the OXPHOS biochemical pathways, and this interaction is expected to result in coadaptation between the nuclear and the mitochondrial genomes (see Rand et al. 2004; Dowling et al. 2008; Gershoni et al. 2009; Hill 2015). Coadaptation involves the mutual evolution of interacting traits via natural selection that enhances their overall function and impact on fitness (Angilletta et al. 2006; Burton and Barreto 2012). The importance of OXPHOS for energy production and the tight functional linkage between the nuclear DNA (nDNA) and mtDNA subunits of the proteins may have broad fitness implications (Burton 1990; Willett and Burton 2003; Ballard et al. 2007; Hoekstra et al. 2013; Bar-Yaacov et al. 2015). When mitochondria are introduced into a novel nuclear background, such

as during a hybridization event between species, incompatibilities between nDNA and mtDNA can result in a reduction of OXPHOS performance, which in turn leads to reduced organismal fitness. For example, mitochondrial function decreased in hybrid offspring of crosses between allopatric populations of copepods (Burton et al. 2006). Incompatibility between nuclear and mitochondrial genomes may also contribute to ecological speciation (Gershoni et al. 2009; Burton and Barreto 2012; Hill 2015).

In addition to high levels of sequence variation, surveys of mtDNA have uncovered many natural examples of introgression of mitochondria across species lines (Toews and Brelsford 2012; Bonnet et al. 2017). Introgression is the movement of genes of one species into another through the process of hybridization. With rapid climate change causing many habitats to shift, many more examples of secondary contact between closely related species should occur, leading to increased opportunities for introgression (e.g., Garroway et al. 2010). These mitochondrial introgression events have important implications for the species involved, but the evolutionary and functional impacts of having a heterospecific form of mitochondria are not well known. Experimental crosses that allow for the expression of mitochondria in novel nuclear backgrounds often show negative effects on mitochondria function (hybrid dysfunction; e.g., Barreto et al. 2015; Chang et al. 2015). Yet in some natural systems of mitochondrial introgression, mitochondrial function displays neutral (chars: Blier et al. 2006; redbelly dace: Deremiens et al. 2015) or positive (warblers: Toews et al. 2013) responses. Studies using cybrid technology, which introduces mitochondria into different genetic backgrounds via tissue culture techniques, also found significant impacts on mitochondrial physiology when mitochondria are expressed in heterospecific nuclear backgrounds (Barrientos et al. 1998; Kazuno et al. 2006; Ji et al. 2012; Latorre-Pellicer et al. 2016).

Hybridization leading to the capture of mitochondria of one species into another species provides a natural system to test the potential role of mitochondria in local adaptation to different environments. These introgression events also provide the opportunity to test for breakdown of coadaptated interactions between proteins of the mitochondrial and nuclear genomes that are expected if disruption of these coadaptations are to function as reproductive isolating mechanisms. Tree lizards (*Urosaurus ornatus*) and long-tailed brush lizards (*Urosaurus graciosus*) have experienced unidirectional exchange of mitochondria (from *U. ornatus* into *U. gracious*) where their ranges overlap in western Arizona (Haenel 2017). *Urosaurus graciosus* specializes in living on the sparse, low vegetation in some of the hottest parts of the North American deserts (fig. 1). *Urosaurus graciosus* lizards survive in these hotter environments in part by maintaining higher body temperatures and water turnover rates (Vitt et al. 1981; Congdon et al. 1982). In contrast, *U. ornatus* has maintained the

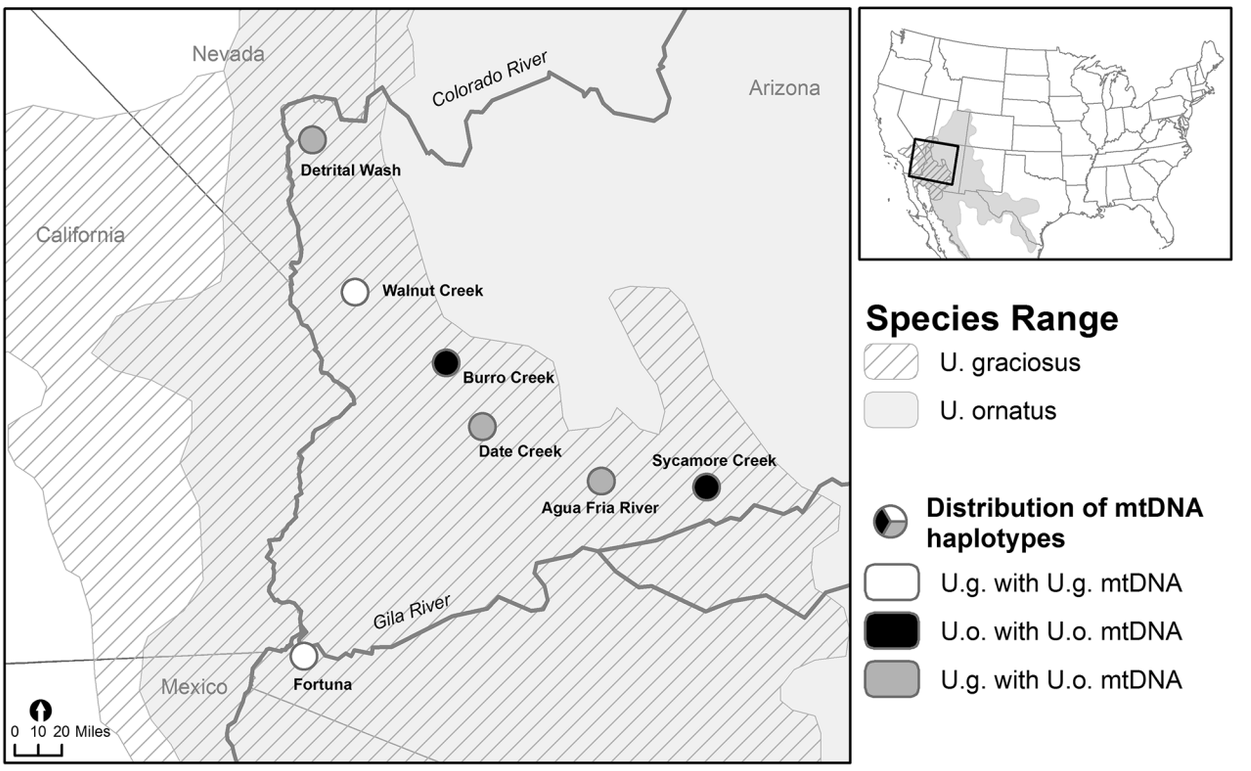

Figure 1. Geographic range map of field locations. Shading represents the range of each species, with circles showing approximate field sampling locations. Coordinates and sample sizes are provided in table 1. U.g. = *Urosaurus graciosus*; U.o. = *Urosaurus ornatus*; mtDNA = mitochondrial DNA.

more ancestral characteristic of occupying a variety of substrates, from trees to rocks (Feldman et al. 2011), across the wide variety of thermal microclimates found within its broad geographic range. The differences in habitat use should impact the energy budgets of these lizards, since temperature has such a large influence on the metabolic rate of small ectotherms (see DeLong et al. 2017). The lizards living in environments with consistently higher temperatures may have consistently higher metabolic rates, which could lead to higher maintenance costs in their energy budgets. For example, higher temperatures caused populations of mottled rock rattlesnakes living at lower elevations to have higher maintenance costs than snakes living at higher elevations and cooler temperatures (Beaupre 1996). Increased temperatures may also increase the flow of electrons through the electron transport chain (ETC) in the mitochondria, providing greater opportunities for production of reactive oxygen species (ROS), which can be damaging to the cell (Murphy 2009; Andriantsitohaina et al. 2012; Schulte 2015) or may interfere with the correct signaling of multiple genes (Aledo 2014; Mason et al. 2016).

The hybrid *Urosaurus* are morphologically indistinguishable from *U. graciosus*, have the nDNA typical of *U. graciosus* based on microsatellite variation, and are found in the same hotter habitats as parental-type *U. graciosus* (Haenel 2017). Furthermore, a gene tree developed with mtDNA sequences placed each population of hybrids into independent, geographically site-specific groups that nested among *U. ornatus* populations (Haenel 2017). Mitochondria in hybrids that coevolved with the nuclear genome of the less heat-tolerant species (*U. ornatus*) have to perform within at least some of the novel nuclear genome of the high-temperature specialist (*U. graciosus*) and under the consistently hotter environmental conditions than where they coevolved with their native nuclear genome. Hybrids should have higher metabolic demands due to living in the more extreme environmental conditions than those generally experienced by parental-type *U. ornatus*. If the *U. ornatus*–type and *U. graciosus*–type mitochondria are coadapted to function with their parental nuclear genomes, we should detect a disruption of function in introgressed lizard mitochondria due to alteration of mitonuclear coadaptations (Pichaud et al. 2012). Coadaptation and epistasis in nuclear and mitochondria gene interactions could lead to hybrid breakdown in individuals with introgressed mitochondria manifested as decreases in the ability to produce ATP and increases in oxidative damage (Barreto et al. 2015; Chang et al. 2015). Because the introgression events in tree lizards appear to be mainly historic, there is the possibility that the introgressed forms of mtDNA have responded to selective forces in the past and represent a new form adapted to these new conditions (*U. ornatus*–type mtDNA expressed in hot conditions). If mitochondrial function is negatively impacted in hybrids, it could act as a reproductive isolating mechanism between these two species by negatively impacting the energy budgets of hybrid individuals and, therefore, viability (Hill 2016).

We used the system of naturally occurring mitochondrial introgression in tree lizards to test whether mitochondria function showed divergence in closely related organisms having different thermal ecologies. We also tested for coadaptation between mtDNA- and nDNA-coded proteins. We compared relative amounts of mitochondria, resting mitochondrial membrane potential, total ATP, ATP production, and oxygen consumption in mitochondria isolated from both parental-type lizards and hybrids containing naturally introgressed mitochondria. Membrane potential is the primary way protons become available for the formation of ATP, and ATP production and oxygen consumption represent key components of OXPHOS reaction (Ballard and Pichaud 2014). Furthermore, we used two temperatures to test for effects of mitochondrial coadaptation on thermal adaptation, as incompatible loci may remain undiscovered under standard temperatures (Koevoets et al. 2012). In all, we addressed the following specific questions. First, is mitochondrial function conserved across species (homospecific forms of mitochondria or parental types)? Second, is mitochondrial function in heterospecific nDNA background (hybrids) different from that in homospecifc (parental-type) nDNA backgrounds? Third, does temperature impact membrane potential the same way in the different types of mitochondria? And fourth, do mitochondria from the species living in hotter environments have lower oxygen consumption for similar rates of ATP production?

Material and Methods

Study Animal

Urosaurus graciosus exhibits a geographic range limited to the Mojave Desert in southern California, northwestern Mexico, and the hotter parts of western Arizona, while *Urosaurus ornatus* is broadly distributed across the southwestern United States and northern Mexico (fig. 1). They display approximately 13% mtDNA sequence divergence and form two independent genetic groups based on microsatellite analyses (Haenel 2017). Divergence of these two species from a common ancestor was estimated at 13.14 million years ago (with a range of 11.34–14.94 million years ago using multiple loci; Hedges et al. 2006, 2015). A total of 59 lizards were sampled for mitochondrial analyses in June 2013 and June 2014 from western Arizona at the sampling locations indicated in figure 1 (see table 1). Sampling locations included two each of parental-type *U. ornatus* (Burro Creek and Sycamore Creek) and *U. graciosus* (Walnut Creek and Fortuna Wash) that had individuals with homospecific (parental) forms of mitochondria and three sites of *U. graciosus* with introgressed mitochondria from *U. ornatus* (hybrids; Detrital Wash, Agua Fria River, and Date Creek Wash). Because of continued drought conditions, individuals at Fortuna Wash were very scarce in 2014 and were not collected. The Date Creek Wash site was added in 2014.

Lizards were captured using a noose. Snout-vent length, hind-limb length, and tail length were measured with a linear ruler (to the nearest 0.5 mm). Mass was measured with a spring scale (to the nearest 0.05 g). Species type was determined on the basis of morphological differences (Haenel 2017). mtDNA type was verified by sequencing parts of the *Cyt-b* or *ND1* mitochondrial genes (see Haenel 2017; GenBank accession numbers are given in table A1) or through polymerase chain reaction using species-specific mtDNA primers (see Lindell and Murphy 2008;

Table 1: Sampling locations and species designations

Site name	Sample size 2013	Sample size 2014	Geographic coordinates	Species designation
Burro Creek, Arizona (BC)	8	4	34°32.555′N, 113°27.004′W	*Urosaurus ornatus* (parental)
Sycamore Creek, Arizona (SYC)	8	4	33°47.799′N, 111°29.665′W	*U. ornatus* (parental)
Fortuna Wash, Arizona (FW)	4	0	32°42.653′N, 114°26.680′W	*Urosaurus graciosus* (parental)
Walnut Creek Wash, Arizona (WALN)	8	4	35°2.176′N, 114°9.619′W	*U. graciosus* (parental)
Agua Fria River, Arizona (AGF)	5	3	33°49.467′N, 112°16.755′W	*U. graciosus* (with *ornatus* mtDNA)
Date Creek, Arizona (DC)	0	4	34°9.160′N, 113°9.966′W	*U. graciosus* (with *ornatus* mtDNA)
Detritial Wash, Arizona (DW)	3	4	35°49.066′N, 114°30.846′W	*U. graciosus* (with *ornatus* mtDNA)

Note. Sample sizes are given for each sampling location by year. Site name abbreviations are given in parentheses. mtDNA = mitochondrial DNA.

fig. A1). Only males were used in this study because females metabolize fats differently across the reproductive cycle (Lacy et al. 2002), and differences in female reproductive state could add uncontrolled variability. Lizards were transported to the laboratory at Elon University, where mitochondrial function was evaluated within 14 d of capture. Lizards were kept in 10-gal terrariums with continuous access to water and lamps that provided free access to environmental temperatures at, above, and below preferred body temperatures for 12 h a day. Crickets (one to three each) were fed to the lizards daily. While lizards were processed as soon as possible after capture, they were neither field-fresh nor fully laboratory acclimated. Common-garden designs are recommended to reduce nongenetic variation among the groups being compared (Garland and Adolph 1991). Common-garden experiments may also expose animals to unnatural conditions, as does full laboratory acclimation. Instead, we pooled data from multiple individuals from multiple locations of each type (the two parental species and their hybrids) sampled during the same season across two years from the same general region (western Arizona).

Mitochondria Isolation

Lizards were euthanized humanely following Institutional Animal Care and Use Committee guidelines. Samples were coded such that experimenters were blind to lizard identity throughout. Livers were removed, weighed, and then rinsed with ice-cold phosphate-buffered saline (1×, −/−). Mitochondria were isolated from fresh livers by mechanical disruption followed by differential centrifugation, as described elsewhere (Lampl et al. 2015). In brief, livers were minced, subjected to four passes using a Dounce homogenizer in isolation buffer (200 mM sucrose, 10 mM Tris/MOPS [3-(*N*-morpholino)propanesulfonic acid; pH 7.4], and 1 mM EGTA [ethylene glycol tetraacetic acid]/Tris), and then spun at 300 *g* (4°C, 10 min). The pellet was rehomogenized and spun again. Supernatants from both spins were combined and then spun at 10,000 *g* (4°C). The pellet was resuspended in isolation buffer, resulting in a crude preparation. Given the small size of lizard livers and multiple planned experiments, further purification was not sought, since doing so would result in a lower amount of final mitochondria available for use. Total protein content was determined (Bio-Rad) as an approximation for mitochondria content, as commonly accepted (MitoSciences 2006; Will et al. 2006; Frezza et al. 2007; Hartwig et al. 2009; Hynes et al. 2009; Rogers et al. 2011; Bharadwaj et al. 2015; Lampl et al. 2015). The amount of mitochondria per liver (mg mito/mg liver) was then calculated for each sample and compared among the three mitochondrial forms (referred to here as "mitotypes"; parental-type *U. graciosus*, parental-type *U. ornatus*, and hybrid for *U. ornatus*–type mitochondria isolated from hybrids that had *U. graciosus* nuclear genomes) using ANOVA. The stats package for R version 3.3 (R Core Development Team 2016) was used for this and all other statistical analyses. Data were log transformed as needed to meet assumptions of the statistical tests.

Evaluation of Mitochondria Function

Before use in experiments, mitochondria were diluted to the appropriate concentration (see below) in cold experimental buffer (125 mM KCl, 10 mM Tris/MOPS [pH 7.4], 5 mM glutamate, 2.5 mM malate, 1 mM potassium phosphate [pH 7.4], and 29 μM EGTA/Tris, filter sterilized). Furthermore, each set of experimental reactions was compared with appropriate controls before data analysis.

Membrane Potential

Resting membrane potential ($\Delta\Psi$) was measured for each cohort using the fluorophore TMRE (tetramethylrhodamine, ethyl ester) by calculating the ratio of the fluorescence of untreated mitochondria to that of mitochondria treated with FCCP (mesoxalonitrile 4-trifluoromethoxyphenylhydrazone) plus valinomycin (Vinsant et al. 2013; Lampl et al. 2015). Mitochondria were diluted to 0.5 mg/mL in cold experimental buffer and then incubated with 1 μM TMRE (tetramethylrhodamine ethyl ester; Invitrogen), with and without 1 μM CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone; Sigma) and 50 nM valinomycin (Sigma), for 10 min at room temperature (RT). Each reaction was then spun at 10,000 *g* (4°C, 5 min) to pellet mitochondria, and 50 μL of the supernatant was added to separate wells of a 96-well plate (Corning). Fluorescence was then measured using an excitation filter of 485 nm (15-nm band pass) and an emission filter of 590 nm (15-nm band pass). All samples were run in duplicate. Ratios of relative fluorescence

units (RFUs) from treated and untreated paired samples were calculated as follows:

$$\frac{\text{RFU}_{\text{mitochondria + CCCP and valinomycin}}}{\text{RFU}_{\text{mitochondria alone}}}.$$

RFUs were then averaged among cohorts. A ratio of >1 indicates that the mitochondria are coupled because on addition of treatment, less fluorophore was taken up due to decreased membrane potential in reaction to the treatment (Lampl et al. 2015). For reactions carried out at 40°C (upper mean field active temperatures measured for *U. graciosus*; Vitt et al. 1981), mitochondria samples were warmed in a water bath for 10 min before addition of TMRE, and reactions were then incubated at 40°C. ANOVA was used to test for differences between mitotypes at each temperature, with membrane potential as the dependent variable and temperature and mitotype as independent variables. Tukey's HSD post hoc test was used for multiple comparisons among mitotypes.

ATP Generation

An ATP Determination Kit (Molecular Probes) was used and modified according to Drew and Leeuwenburgh (2003). In brief, the luminescent signals for eight ATP standards (0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, and 100 μM) were used to extrapolate ATP concentration in experimental samples on the basis of luminescence. For each set of reactions, background luminescence of the reaction mixture was taken for standards, controls, and experimental reactions (before addition of mitochondria), which was then subtracted from the final luminescent values. Control reactions included experimental reactions set up with the addition of oligomycin to ensure that ATP production could be inhibited and therefore that the mitochondria were functional. Total ATP for each experimental sample was also measured before addition of ADP substrate and subtracted out so that only new ATP produced was quantified. ATP production at both RT and 40°C (under heat stress) was then measured by adding mitochondria and ADP to each reaction. Each reaction was plated in quadruplicate, with duplicates either with or without the addition of oligomycin. Total ATP was compared among mitochondrial types using ANOVA with micromoles per liter of ATP per microgram of mitochondria as the dependent variable. For ATP generation reactions carried out at 40°C, mitochondria samples were warmed in a water bath for 10 min before the reactions, and then reactions were carried out in an EnSpiron multimode plate reader (PerkinElmer) set to 40°C. The statistical approach used to test ATP production was the same as that used for membrane potential.

Oxygen Consumption

Oxygen consumption in freshly isolated liver mitochondria was determined using a MitoXpress Xtra Hs assay (Luxel) according to the manufacturer's protocol but modified according to Hynes et al. (2006) and Will et al. (2006). In brief, mitochondria were diluted in experimental buffer to 6 mg/mL and then plated in a 96-well plate with equal volumes with 0.05 M glutamate and malate in Tris/MOPS (pH 7.4) with or without 1.65 mM ADP, representing states 3 and 2, respectively (Lanza and Nair 2009; Irwin et al. 2011; Dunn and Pinkert 2012), and 2× volume fluorescent probe. Each reaction was topped with heavy mineral oil, supplied in the kit, to exclude ambient oxygen, and the plate was read in the plate reader equilibrated to 30°C. Fluorescent measurements (380/650-nm excitation/emission) were taken at 30°C in kinetic mode every 1.5 min with a time delay of 30 μs and a measurement window of 100 μs for 45 min total. To ensure gas and temperature equilibration of samples at the start of the assay, all of the dispensing steps were carried out at 30°C using prewarmed solutions. Analysis of fluorescence intensity in each well over time was analyzed to determine the rates of oxygen consumption based on the known relationship between probe fluorescence and oxygen concentration (Lakowicz 1999; Hynes et al. 2006). Fluorescent readings were first plotted against time and compared with control reactions (buffer plus glutamate and malate, with and without ADP plus probe and oil) to ensure that any change in RFUs in experimental reactions was due to a reaction of mitochondria, to determine the maximal RFUs for the set of reactions, and to ascertain the time point at which the signal leveled out. Fluorescent traces for experimental reactions were then transformed into oxygen concentration profiles by means of the following formula (Hynes et al. 2006):

$$[\text{O}_2]t = \frac{[\text{O}_2]_\text{a} \times I_\text{a} \times (I_\text{o} - I_{(t)})}{I_{(t)} \times (I_\text{o} - I_\text{a})},$$

where $[\text{O}_2]_\text{a}$ is oxygen concentration in air-saturated buffer (235 μM at 30°C) and $I_{(t)}$, I_a, and I_o are the fluorescent signal of the probe at time t, signal in air-saturated buffer (baseline signal without enzyme), and signal in deoxygenated buffer (maximal signal), respectively. Rates of change of dissolved oxygen (μM/min) were extrapolated from the initial slopes of these decreasing oxygen concentration profiles for each sample. Slopes were then aggregated for each mitotype for comparison of rate of consumption. Respiratory control ratio (RCR) values for each sample were determined in the linear region of the oxygen consumption curves (Hynes et al. 2006; Irwin et al. 2011; Dunn and Pinkert 2012; Ma et al. 2017) and calculated by dividing state 3 values by state 2 values. All reactions were run in duplicate. The statistical approach used to test oxygen consumption was the same as that used for ATP production and membrane potential.

Results

One common response to repeated metabolic stress is the production of more mitochondria, leading to a greater overall output of ATP (e.g., Weibel et al. 2004; Holloszy 2008). Therefore, we first tested for differences in the mitochondria content of liver tissue in parental type and hybrid lizards. The total amount of mitochondria in livers among the three mitochondrial types was not significantly different, as measured by total protein in freshly isolated mitochondria samples after removing the effect of liver mass (fig. 2; ANOVA: $F_{2,47} = 0.893$, $P = 0.416$; $n = 14$, 22,

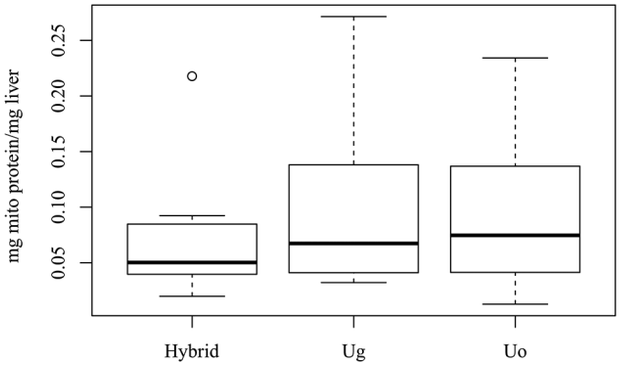

Figure 2. Quantity of isolated mitochondria from each mitochondria type. The midlines of box plots indicate the median quantity of mitochondria, the upper and lower limits of each box show the third and first quartiles, and the whiskers indicate minimum and maximum values. Mitochondria were isolated from livers and quantified, and then values were adjusted per the amount of liver tissue. There was no significant difference in the amount of mitochondria isolated from each mitochondrial type. Labels indicate *Urosaurus graciosus* parental type (Ug), *Urosaurus ornatus* parental type (Uo), and hybrid or introgressed *U. ornatus* mitochondria in *U. graciosus* nuclear background.

and 14 for *Urosaurus graciosus*, *Urosaurus ornatus*, and hybrid forms, respectively).

Assessment of Mitochondrial Resting Membrane Potential

Quantification of resting membrane potential can be informative when comparing mitochondria fitness among different groups. We used mitochondria loaded with TMRE and treated with uncouplers to assess membrane potential. Mitochondria that have intact membranes and are biochemically functional should respond to treatment with FCCP plus valinomycin by decreasing membrane potential. For functional comparison, we calculated the ratio of untreated to treated mitochondria for each mitochondrial type (*U. graciosus*, *U. ornatus*, and hybrid; see "Material and Methods"). We found that temperature significantly impacted membrane potential (fig. 3; $F_{1,43} = 34.651$, $P = 5.35\text{E}{-}07$). Although no overall significant difference was found among the mitochondrial types (fig. 3; ANOVA: $F_{2,43} = 0.218$, $P = 0.8051$; $n = 7$, 8, and 9 at RT and $n = 7$, 8, and 11 at 40°C for *U. graciosus*, *U. ornatus*, and hybrid forms, respectively), there was a marginally significant interaction for temperature and type of mitochondria (fig. 3; ANOVA: $F_{2,43} = 2.909$, $P = 0.0653$). Temperature impacted membrane potential differently in the different types of mitochondria. Both parental types showed significant decreases with temperature, while the introgressed (hybrid) form of mitochondria did not (fig. 3; Tukey's HSD post hoc: $P_{\text{adjusted}} < 0.001$ for *U. graciosus*, $P_{\text{adjusted}} = 0.020$ for *U. ornatus*, $P_{\text{adjusted}} = 0.312$ for hybrid).

ATP Generation in Isolated Mitochondria

Primary assessment of total ATP for each sample was measured as the starting point from which to measure production. We found that initial amounts of ATP were the same for each type of mitochondria (ANOVA: $F_{2,27} = 2.956$; Tukey's HSD post hoc: $P_{\text{adjusted}} > 0.077$; $n = 7$, 10, and 13 for *U. graciosus*, *U. ornatus*, and hybrid, respectively). Given this result, any differences in ATP production should be due to changes in mitochondrial processes themselves. Temperature and mitochondrial type were examined, and both significantly impacted ATP production (fig. 4; $F_{1,83} = 7.467$, $P = 0.0077$ for temperature; $F_{2,83} = 8.446$, $P = 0.0004$ for mitotype; $n = 16$, 23, and 18 at RT and $n = 7$, 13, and 12 at 40°C for *U. graciosus*, *U. ornatus*, and hybrid, respectively). Specifically, ATP production by hybrid mitochondria was significantly higher than mitochondria of either parental type (fig. 4; Tukey's HSD post hoc: $P_{\text{adjusted}} = 0.0053$ for hybrid × *U. graciosus*, $P_{\text{adjusted}} = 0.0009$ for hybrid × *U. ornatus*, $P_{\text{adjusted}} = 0.99851$ for *U. ornatus* × *U. graciosus*). However, the interaction between temperature and mitotype was not significant (fig. 4; $F_{2,83} = 0.314$, $P = 0.7314$), indicating that ATP production of each mitotype responded to temperature in a similar manner.

Oxygen Consumption

Respiration with and without ADP—states 3 and 2, respectively—was measured. Fluorescence, which increases as oxygen concentration decreases (Hynes et al. 2006; Will et al. 2006), was plotted for each isolated mitochondrial sample; the linear portion of the line was used to calculate RCR, and data were transformed to calculate oxygen consumption rate (fig. 5). RCR values were lower in hybrids than in *U. ornatus* parental-type mitochondria (fig. 5C; mean RCR: 6.615 for *U. graciosus*, 3.754 for hybrid, 6.963 for *U.*

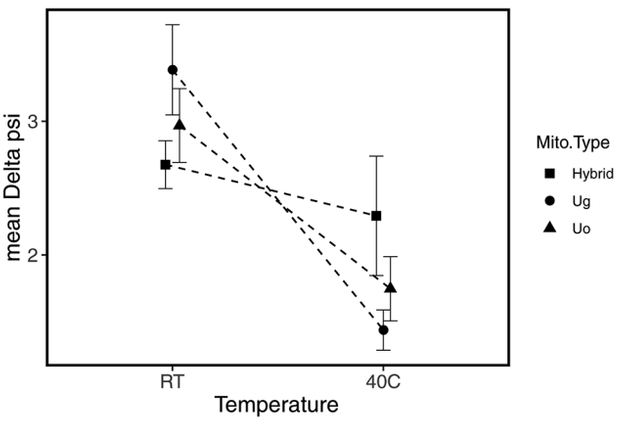

Figure 3. Resting membrane potential for each mitochondrial type measured at room temperature (RT) and 40°C. Mean relative fluorescence unit (RFU) values (±1 SE) of resting membrane potential are plotted for each mitochondrial type at two temperatures. No significant difference among the three types was found at RT. High temperature (40°C) significantly affected *Urosaurus graciosus* parental type (Ug; high-temperature specialist) and *Urosaurus ornatus* parental type (Uo) but not the introgressed forms (hybrid; *U. ornatus*–type mitochondria in *U. graciosus* nuclear background). Lines were added to aid visual comparison between each set of samples at the two temperatures.

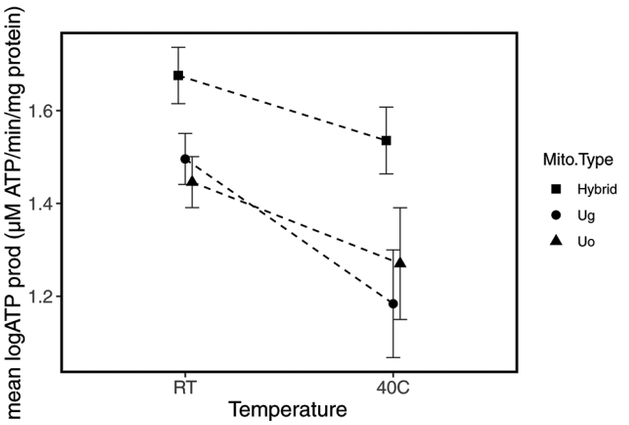

Figure 4. Quantification of ATP production among different mitochondrial types at room temperature (RT) and 40°C. Mean ATP production (±1 SE) is shown for each mitochondrial type. Introgressed forms of mitochondria (hybrid) had significantly higher ATP production than either parental type, *Urosaurus graciosus* (Ug) and *Urosaurus ornatus* (Uo). All three had a similar decrease in ATP production at 40°C. Lines are present only to aid in visual comparison between each set of samples at the two temperatures.

ornatus; Tukey's HSD post hoc: $P_{\text{adjusted}} = 0.1141$ for hybrid × *U. graciosus*, $P_{\text{adjusted}} = 0.0458$ for hybrid × *U. ornatus*, $P_{\text{adjusted}} = 0.9685$ for *U. ornatus* × *U. graciosus*; $n = 10$, 13, and 15 for *U. graciosus*, *U. ornatus*, and hybrid, respectively). There was also a significant effect of mitochondrial type, but not temperature, on oxygen consumption (fig. 6; $F_{1,55} = 0.523$, $P = 0.4727$ for temperature; $F_{2,55} = 3.125$, $P = 0.0518$ for mitotype; $F_{2,55} = 0.373$, $P = 0.6906$ for temperature × mitotype; $n = 10$, 13, and 15 at RT and $n = 4$, 11, and 8 at 40°C for *U. graciosus*, *U. ornatus*, and hybrid, respectively). Post hoc comparisons found the difference to be between parental types of mitochondria, with *U. graciosus* parental-type mitochondria consuming significantly less than *U. ornatus* parental-type mitochondria (fig. 6; Tukey's HSD post hoc: $P_{\text{adjusted}} = 0.1338$ for hybrid × *U. graciosus*, $P_{\text{adjusted}} = 0.8347$ for hybrid × *U. ornatus*, $P_{\text{adjusted}} = 0.0475$ for *U. ornatus* × *U. graciosus*).

Discussion

Introgression of mitochondria between two sister species of lizards with consistent unidirectional backcrossing allowed us not only to test functional divergence between mitochondria of a high-temperature specialist (*Urosaurus graciosus*) and a more "thermal generalist" (*Urosaurus ornatus*) but also to compare the function of mitochondria of the thermal generalist expressed in the nuclear background of the high-temperature specialist (hybrids). This three-way comparison, including multiple populations of each species, was important because some divergence in physiological traits would be expected just by virtue of them being different species (Garland and Adolph 1994). Overall, we found that some components of mitochondrial func-

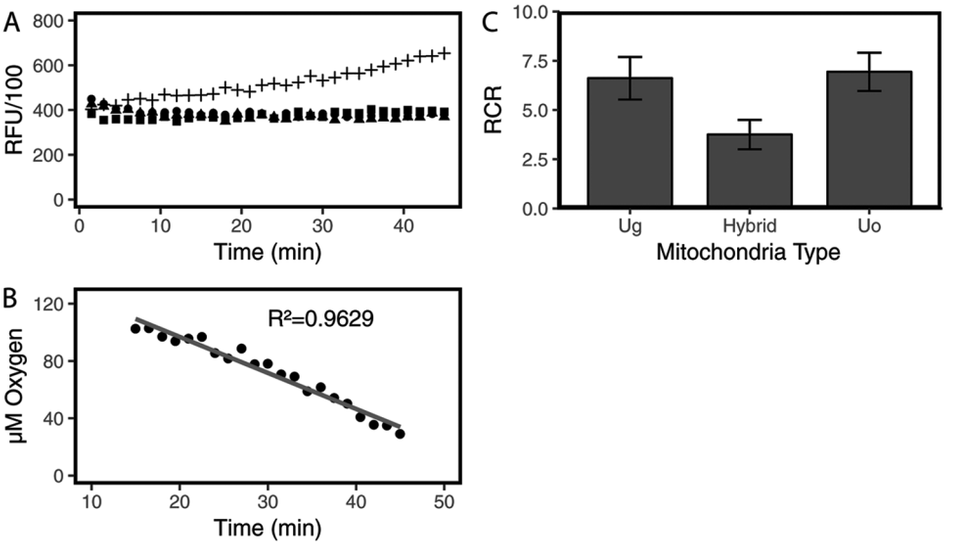

Figure 5. Raw and transformed oxygen concentration data. *A*, Sample of raw data from oxygen consumption assays. Buffer-only control reactions and reactions containing isolated mitochondria were treated with glutamate and malate (G/M), with or without ADP, and fluorescence was measured over time. As the concentration of oxygen decreases due to it being consumed, fluorescence increases. Buffer alone (G/M, shown by circles, and G/M plus ADP, shown by triangles) as well as mitochondria with G/M but without ADP (shown by squares) were relatively flat, while mitochondria treated with G/M plus ADP (shown by plus signs) consumed oxygen. *B*, Oxygen concentration over time extrapolated from the raw data (for calculation specifics, see "Material and Methods"). Shown are the values from mitochondria treated with G/M plus ADP from *A*. *C*, Respiratory control ratio (RCR) values for the three types of mitochondria at room temperature. Introgressed forms (hybrid) have lower values than the *Urosaurus ornatus* parental type (Uo), while values for the *Urosaurus graciosus* parental type (Ug) are not significantly different from those for the *U. ornatus* parental type or hybrids. Means (±1 SE) are shown. RCR = respiratory control ratio; RFU = relative fluorescence unit. A color version of this figure is available online.

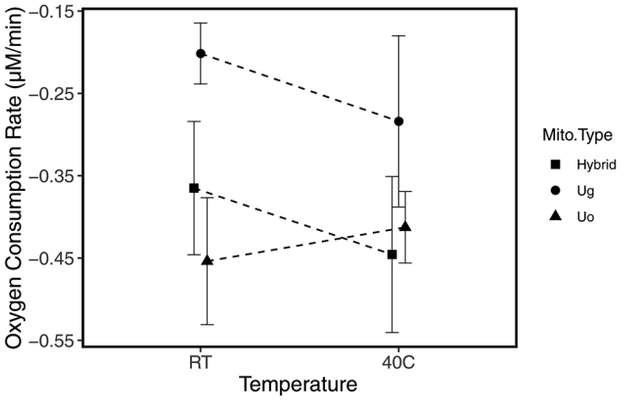

Figure 6. Oxygen consumption rates compared among three types of mitochondria at room temperature (RT) and 40°C. Mean oxygen consumption rates (±1 SE) are shown for each mitochondrial type. *Urosaurus graciosus* (Ug) and *Urosaurus ornatus* (Uo) parental types were significantly different from each other. Introgressed forms (hybrid) were intermediate to both parental types and not significantly different from either. Lines are present only to aid in visual comparison between each set of samples at the two temperatures. RCR = respiratory control ratio; RFU = relative fluorescence unit.

tion were conserved between species, while others were not. Furthermore, introgressed forms of mitochondria showed divergence from both parental forms in multiple aspects of mitochondrial function.

The first aspect of mitochondrial function examined was resting membrane potential, which represents the energy available for the production of ATP (Brand et al. 1991). Membrane potential was similar across all three forms (fig. 3), which would be expected if mitochondrial function is evolutionarily conserved (Wolff et al. 2014). Furthermore, membrane potential of both homospecific forms also decreased to a similar degree at the higher temperature (fig. 3). There are numerous reasons for such a decrease in membrane potential at increased temperatures (Lee and Gear 1974; Blicher et al. 2009), all of which ultimately result in increased leaking of protons across mitochondrial membranes. In small ectotherms, decreased membrane potential at higher temperatures could possibly slow ATP production rather than having the expected ATP production increase due to thermodynamics of chemical reactions alone (DeLong et al. 2017). Slowing ATP production as a response to increased temperatures could reduce overall respiratory maintenance costs as well as decrease the amount of ROS being produced.

Interestingly, analysis of membrane potential response to increased temperature indicated an interaction between mitochondrial source and temperature. Specifically, the membrane potential of parental-type mitochondria decreased significantly at the higher temperature but did not change significantly in introgressed mitochondria. Therefore, the membrane potential of the introgressed mitochondria appears to have less plasticity in response to higher temperatures. This type of interaction between temperature and cytoplasm has been shown to affect incompatibilities in other hybrids (Willett and Burton 2003; Demuth and Wade 2007; Arnqvist et al. 2010). The lack of significant change in membrane potential by the introgressed forms of mitochondria but not the homospecific forms indicates the presence of a mitochondrial genotype × nuclear genotype × environment interaction. Such a result would be expected if there was a breakdown in coevolved interactions between mtDNA and nDNA products used in the mitochondria of these species (Sloan et al. 2017).

More than 90% of the body's energy comes from production of ATP in the mitochondria, thereby functionally linking mitochondria to an individual's overall energy budget (see Gershoni et al. 2009; Ballard and Melvin 2010). Therefore, the next aspect of mitochondrial function examined was ATP production. Not only was ATP production similar among the two parental types, but both also decreased production at 40°C (fig. 4) in a manner similar to that found for membrane potential. In contrast, introgressed forms of mitochondria had significantly higher rates of ATP production at both temperatures, indicating a mitochondrial genotype × nuclear genotype interaction. Taking these results together, individuals with introgressed mitochondria make more ATP with membrane potentials similar to those of parental types of mitochondria. One explanation for these results could be heterosis, where hybrid forms show greater performance than parental types. However, this is an unlikely explanation for this result, since heterosis is common for F_1's (Edmands 1999), but further backcrossing should uncover hidden dysfunctions, especially in F_2's (Burton et al. 2006). Furthermore, no *U. ornatus* parental types are currently known to be present in the locations where hybrids were sampled, and all individuals previously sampled from these populations were also found to be backcrossed into *U. graciosus* parental genotypes (Haenel 2017). Higher ATP production in hybrids may therefore represent a compensatory response to maintain stable cellular function due to mitonuclear discordance.

Alternatively, higher ATP production could signify a maladaptive response to the high-temperature environments in which hybrid individuals are living. At higher temperatures, ATP production increases up to a point in small ectotherms, but efficiency then decreases (Schulte 2015). As such, maintaining higher ATP production (and membrane potential) could result in higher maintenance costs in the overall energy budget of hybrids. This interpretation may then account for the smaller body sizes observed for some *Urosaurus* lizards with introgressed mtDNA (Haenel 2017). In addition, higher rates of ATP production may also lead to greater ROS production as a by-product of OXPHOS (Nohl and Hegner 1978; Nohl 1986; Barreto and Burton 2013). Increased ROS was observed in cybrid nematodes containing heterospecific forms of mitochondria (Chang et al. 2015). Potential evolutionary mechanisms for observed differences in ATP production may include modification of nuclear and mitochondrial protein interactions within this part of the OXPHOS pathway, changes in regulatory elements, and differential gene regulation (Davies et al. 2012; Barreto et al. 2015).

To provide insight into energy metabolism, respiratory control and rate of oxygen consumption were analyzed in

isolated mitochondria. RCR values capture the main function of mitochondria, the ability to make ATP in response to an influx of ADP. These calculated values not only signify the overall health of isolated mitochondria samples but also allow comparison of responses among different sample types. RCR values obtained were lower in introgressed forms of mitochondria than in either parental type (fig. 5). This difference may represent mitochondrial dysfunction in hybrids (Brand 2011) and indicates a biological difference between homospecific and heterospecific forms of mitochondria. Quantifying absolute rates of respiration, also known as oxygen consumption rates during OXPHOS, can give more direct insight into the disparities of mitochondrial function (Brand and Nicholls 2011) and therefore was examined. We found that *U. graciosus* parental-type mitochondria had significantly lower oxygen consumption rates than *U. ornatus* parental-type mitochondria, while introgressed forms of mitochondria had a mean oxygen consumption rate between that of the parental types (fig. 6). One explanation for these results is that even though oxygen consumption is a good indicator of flux through the ETC, it may not be directly correlated with ATP production due to such factors as different rates of proton leakage (Schulte 2015). If enzymes were not subject to genetic incompatibilities and the hybrids were F_1's, the result of intermediate levels of activity would be expected (e.g., Pasdar et al. 1984), and because hybrids were not likely to be F_1's (see above), a more complex process could be indicated. Furthermore, not all energy used by the mitochondria goes to ATP production (Brand 2005), and since ATP production was similar between parental types, any energy savings should be in another part of the OXPHOS cycle. One possible place is in the maintenance of membrane potential, which would be consistent with our observations. *Urosaurus graciosus* parental-type mitochondria may have a more porous membrane, possibly due to the hotter body temperatures they experience or, alternatively, modified uncoupler proteins. Consequently, less oxygen may need to be consumed by *U. graciosus* parental-type mitochondria to provide similar amounts of ATP as *U. ornatus* parental-type mitochondria, with fewer electrons moving through the mitochondrial ETC. Since electron flow through the ETC inevitably produces ROS (Murphy 2009) and ROS formation is positively related to the proton-motive force (Korshunov et al. 1997; Brand 2000; Salin et al. 2015), this scenario could also beneficially decrease the amount of ROS production.

Mitochondrial efficiency (the P/O ratio, defined as moles of ATP synthesized per moles of $O(\frac{1}{2}O_2)$ used; Brand and Nicholls 2011) is another parameter often measured in isolated mitochondria. Since ATP production and oxygen consumption experiments were carried out in separate reactions, we were not able to directly estimate P/O ratios. However, *U. graciosus* parental-type mitochondria had significantly lower oxygen consumption than *U. ornatus* parental-type mitochondria, but they did not have significantly different levels of ATP production. These results imply that the heat-tolerant *U. graciosus* may have greater efficiency than the less heat-tolerant *U. ornatus*, which might be an important factor for energy conservation of a small ectotherm living at high temperatures.

The pattern of unidirectional introgression in *Urosaurus* lizards was consistent with expectations of past population expansions hypothesized from current population genetic patterns (Haenel 2017). However, unidirectional success of crosses could also be supported by a variety of other adaptive and nonadaptive mechanisms (reviewed in Sloan et al. 2017), including asymmetric genetic incompatibilities such as Dobzhanski-Muller interactions (e.g., Bordenstein and Drapeau 2001). Since the mitochondrial genome is maternally inherited, if epistatic interactions of the mitochondria with nDNA are present, then only one hybrid cross direction should result in the production of dysfunctional hybrids (Turelli and Moyle 2007). In contrast, when separation of coevolved mitochondrial and nuclear loci causes hybrid dysfunction, there should be symmetrical effects because coevolved alleles at two (or more) loci should exist in both parental populations. All hybrids, regardless of the direction of cross, then should see deleterious effects of separating the coevolved alleles of epistatic genes (Chang et al. 2015). The neutral accumulation of multiple mitonuclear Dobzhanski-Muller interactions could also produce a symmetrical effect (Turelli and Orr 2000; Turelli and Moyle 2007; Chang et al. 2015). The mixed response we found in different parts of the OXPHOS process indicates that a complex set of processes is likely at work.

Natural selection on introgressed forms of mitochondria could account for the different responses of mitochondria we observed. Selection may be acting in hybrid populations to overcome the costs associated with the functioning of heterospecific mitochondria in a novel genomic environment. Genetic drift in hybrid populations could also cause independent responses. In addition to energy production, mitochondria also function to integrate signals controlling lipid metabolism, apoptosis, development rate, and cell cycle control (Chang et al. 2015). Thus, other forms of mitonuclear epistasis could be impacting the fitness of these hybrids beyond the ETC genes and could even potentially involve variation in noncoding regions (e.g., Nam and Kang 2001; Karlok et al. 2002; Dowling et al. 2007; Meiklejohn et al. 2007; Ellison and Burton 2008*a*).

By synthesizing data from multiple aspects of mitochondrial function ($\Delta\Psi$, ATP production, RCR, and oxygen consumption), we found that while membrane potential and ATP production were conserved between mitochondria of the two species (homospecific forms), oxygen consumption had diverged. Furthermore, introgressed mitochondria showed divergent function relative to both parental forms. These differences among mitochondria are consistent with the breakdown of coevolved mitonuclear interactions in introgressed forms. The changes in mitochondrial function observed in hybrids are significant and have the potential to act as a reproductive isolating mechanism where populations of these two species overlap geographically.

Acknowledgments

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APPENDIX

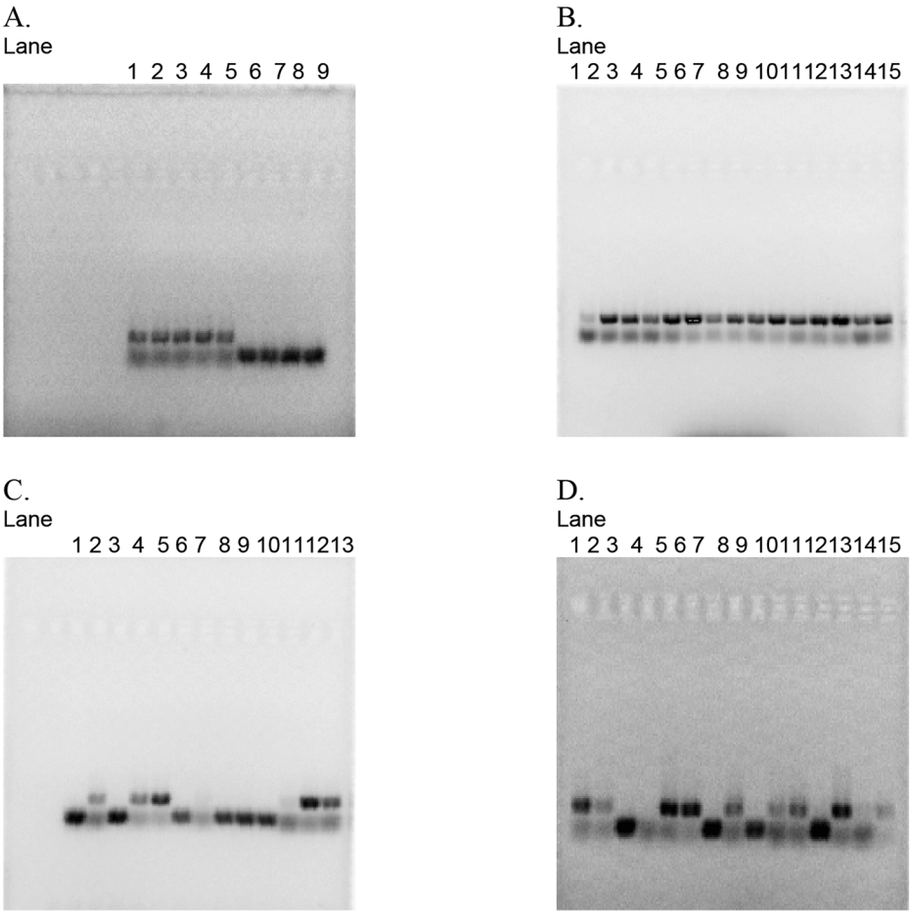

Figure A1. Photos from 1% agarose gel run for 30 min in 1× TAE (Tris base, acetic acid, and EDTA) buffer at 60 V showing polymerase chain reaction (PCR) products of three-primer *ND1* PCRs (forward, 5′-ACGTGACTTAGTTAGGGTGGC-3′; Rev_Ug, 5′-GTAGGGTAAGGAAG GCGACG-3′; Rev_Uo, 5′-AAGTGCAAGTATAAATAGAAGT-3′). A single smaller fragment represents the *Urosaurus graciosus* mitochondrial DNA haplotype, while the presence of a larger band represents the *Urosaurus ornatus* haplotype. Localities are as in table A1. Lane numbers show sample IDs as follows: in *A*, 1 = AGF0551, 2 = SYC0312, 3 = DW0303, 4 = BC0245, 5 = BC0134, 6 = WALN0031, 7 = WALN0024, 8 = WALN0022, 9 = WALN0015; in *B*, 1 = SYC0154, 2 = SYC0056, 3 = SYC043(34), 4 = SYC0(34)53, 5 = DW6002, 6 = DC4430, 7 = DW4300, 8 = DW4200, 9 = DC4015, 10 = DC4001, 11 = SYC3103, 12 = AGF1250, 13 = AGF1140, 14 = BC1102, 15 = BC1053; in *C*, 1 = WALN1305, 2 = BC1240, 3 = FW1204, 4 = SYC1064, 5 = BC1034, 6 = WALN1013, 7 = not available, 8 = FW0535, 9 = WALN0521, 10 = WALN0451, 11 = BC0354, 12 = DW0252, 13 = DW0214; in *D*, 1 = BC4440, 2 = SYC4104, 3 = FW4043, 4 = SYC3504, 5 = BC3344, 6 = AGF3043, 7 = WALN3033, 8 = SYC3024, 9 = WALN2530, 10 = AGF2440, 11 = AGF2240, 12 = WC2102, 13 = BC1034, 14 = BC2644, 15 = AGF1340.

Table A1: GenBank accession numbers for mitochondrial DNA (mtDNA) sequences confirming mtDNA haplotypes

Site	Accession nos.
AGF	MH006971, MH006985, MH006986, MH006987, MH007000
BC	MH006972, MH006978, MH006980, MH006981
DC	MH006970, MH006983, MH006984, MH006999
DW	MH006988, MH006989
FW	MH006996, MH006998
SYC	MH006976, MH006977, MH006979
WALN	MH006990, MH006993, MH006994, MH006995, MH006997, MH006969, MH006973, MH006975, MH006974

Note. Population IDs are as follows: AGF = Agua Fria River; BC = Burro Creek; DC = Date Creek; DW = Detritial Wash; FW = Fortuna Wash; SYC = Sycamore Creek; WALN = Walnut Creek Wash.

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