Integrative and Comparative Biology

Integrative and Comparative Biology, volume 59, number 4, pp. 925–937
doi:10.1093/icb/icz124

Society for Integrative and Comparative Biology

SYMPOSIUM

Mitochondrial Ecophysiology: Assessing the Evolutionary Forces That Shape Mitochondrial Variation

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From the symposium "Beyond the powerhouse: integrating mitonuclear evolution, physiology, and theory in comparative biology" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2019 at Tampa, Florida.

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Synopsis The mitonuclear species concept hypothesizes that incompatibilities between interacting gene products of the nuclear and mitochondrial genomes are a major factor establishing and maintaining species boundaries. However, most of the data available to test this concept come from studies of genetic variation in mitochondrial DNA, and clines in the mitochondrial genome across contact zones can be produced by a variety of forces. Here, we show that using a combination of population genomic analyses of the nuclear and mitochondrial genomes and studies of mitochondrial function can provide insight into the relative roles of neutral processes, adaptive evolution, and mitonuclear incompatibility in establishing and maintaining mitochondrial clines, using Atlantic killifish (*Fundulus heteroclitus*) as a case study. There is strong evidence for a role of secondary contact following the last glaciation in shaping a steep mitochondrial cline across a contact zone between northern and southern subspecies of killifish, but there is also evidence for a role of adaptive evolution in driving differentiation between the subspecies in a variety of traits from the level of the whole organism to the level of mitochondrial function. In addition, studies are beginning to address the potential for mitonuclear incompatibilities in admixed populations. However, population genomic studies have failed to detect evidence for a strong and pervasive influence of mitonuclear incompatibilities, and we suggest that polygenic selection may be responsible for the complex patterns observed. This case study demonstrates that multiple forces can act together in shaping mitochondrial clines, and illustrates the challenge of disentangling their relative roles.

Introduction

Mitochondria are the primary source of adenosine triphosphate (ATP) in eukaryotic cells, act as a key signaling hub in responses to environmental stressors, and can even control cell life and death (Kasahara and Scorrano 2014; Vakifahmetoglu-Norberg et al. 2017). Because of these important roles, changes in mitochondrial function have been implicated in organismal adaptations to their environments (Ballard and Rand 2005). Of the ~1000 proteins in the mitochondrial proteome (Smith and Robinson 2016; Höß et al. 2017) only 13 are present in the mitochondrial genomes of bilaterian animals (Lavrov and Pett 2016). All of these mitochondrially-encoded proteins are part of the multi-subunit protein complexes that form the electron transport system (ETS) and the ATP synthase, which together form the oxidative phosphorylation apparatus of the cell. These 13 mitochondrially-encoded proteins interact directly with ~76 nuclear encoded proteins allowing the oxidative generation of ATP. In addition, mitochondrial genomes of bilaterian animals encode two ribosomal RNAs and 22 transfer RNAs (tRNAs), which must work with nuclear-encoded proteins to facilitate mitochondrial translation (Lavrov and Pett 2016). As a result, many important mitochondrial functions, such as oxidative phosphorylation and mitochondrial transcription and

Advance Access publication July 8, 2019

translation, are dependent on interactions between nuclear and mitochondrially encoded gene products. It is critical to maintain these functional interactions between nuclear and mitochondrial gene products to support organismal performance.

Interactions between nuclear-encoded and mitochondrially-encoded gene products have the potential to lead to interesting co-evolutionary dynamics between these two genomes (Rand et al. 2004; Gershoni et al. 2009; Dowling 2014). This phenomenon has led to the proposal of the mitonuclear species concept (Hill 2016), which posits that breakdown of mitonuclear interactions in hybrids is a key component of post-zygotic isolation that can lead to speciation. In brief, the theory proposes that the rapid accumulation of mitochondrial mutations, due to the higher mutation rate in the mitochondrial genome, must be compensated by mutations in the nuclear genome that maintain mitochondrial function. Over time, this will lead to divergence between isolated taxa in both the mitochondrial genome and in the nuclear genome to maintain these functional interactions within each taxon. When two previously isolated taxa come into contact, breakdown of these co-evolved nuclear–mitochondrial interactions in hybrids can form a potent post-zygotic isolating barrier that maintains species boundaries. Although there is good evidence to suggest that mitonuclear incompatibility may act as an isolating barrier in some taxa (Burton et al. 2013; Wolff et al. 2016; Barreto et al. 2018; Hill et al. 2019; Morales et al. 2018), whether this mechanism is as or more important than the many other isolating barriers (both pre-zygotic and post-zygotic) that can separate species remains an open question.

A key prediction of the mitonuclear species concept is that there should be sharp disjunctions in mitochondrial DNA (mtDNA) sequence between species, and where closely related species meet at hybrid zones both mtDNA and nuclear genes that are involved in mitochondrial processes should show greatly reduced introgression across the hybrid zone (Hill 2017). As a result of these interactions, there should be strong and pervasive signals of negative epistasis in patterns of sequence variation in the nuclear genome. In addition, failure of mitochondrial processes should be observable in hybrids, particularly in the F2 generation and beyond. However, there have been relatively few tests of the mitonuclear species concept that address the full suite of these predictions, and particularly the key prediction of mitochondrial failure in hybrids (although see (Barreto and Burton 2013; Burton et al. 2013; Baris et al. 2017)). Instead, the majority of the data supporting the mitonuclear species concept come from observations of patterns of sharp disjunctions in mitochondrial genotype between taxa (Hill 2016). Sharp disjunctions in mitochondrial genotype can be produced by a variety of processes, and not just mitonuclear incompatibility. Therefore, these studies provide only circumstantial support for the mitonuclear species concept.

There are three main scenarios under which steep clines in mtDNA could arise. The first involves purely neutral processes such as recent secondary contact between isolated populations that have diverged as a result of genetic drift or genetic surfing during range expansion (Excoffier et al. 2009). A second scenario that can produce steep mitochondrial clines is local adaptation of mitochondrially-encoded proteins along an environmental gradient (Irwin 2012). The final scenario that can produce steep clines in mtDNA, and the one that is highlighted in the mitonuclear species concept, is mitonuclear incompatibilities that cause hybrid breakdown when previously isolated taxa come into contact.

Although all of these processes can produce steep mitochondrial clines, it may be possible to distinguish among them based on their predicted effects on patterns in the frequencies of alleles in the nuclear genome and in the likelihood that they would be associated with effects on mitochondrial function. For example, recent secondary contact between previously isolated forms that have diverged neutrally as a result of genetic drift should produce spatially correlated patterns of genetic variation in both the nuclear and mitochondrial genomes. However, these processes can also produce discordant patterns in the nuclear and mitochondrial genomes as a result of the lower effective population size of mtDNA, and other processes such as sex-biased dispersal, which can lead to steeper clines in mtDNA than in nuclear DNA (Toews and Brelsford 2012). Similarly, genetic surfing can also result in steeper clines in the mitochondrial genome than in the nuclear genome because of the lower effective population size of the mitochondrial genome. In both of these cases, some steep nuclear DNA clines would be expected, but they would likely be randomly distributed across the nuclear genome, rather than being associated with nuclear-encoded proteins involved in mitochondrial function. In addition, these neutral processes would be less likely to result in differentiation of mitochondrial function, given the strong purifying selection acting on mitochondrial ATP production.

The second scenario that has the potential to result in steep clines in mtDNA along an environmental gradient is local adaptation of mitochondrial processes (Irwin 2012). In this case, steep clines would be likely to occur in mtDNA and in nuclear-encoded but mitochondrially localized genes, rather than being randomly distributed through the nuclear genome, as would be expected for neutral processes. Another key hallmark of local adaptation shaping steep clines in mtDNA would be differentiation of mitochondrial function between the locally adapted taxa. Local adaptation of mitochondrial function that results in steep clines in mtDNA represents a specific case of exogenous (environment dependent) selection acting on the mitochondrial genome. If this functional differentiation in genes encoded in the mitochondrial genome is not accompanied by functional differentiation in nuclear-encoded mitochondrial genes, then the mitochondrial properties of hybrids would be expected to be a reflection of their mitochondrial genome alone. Alternatively, if functional differentiation also occurs in nuclear encoded mitochondrial genes, then advanced generation hybrids may display a range of phenotypes encompassing that of both parental populations, or they may even display a phenotype more extreme than that of the parental populations, a phenomenon known as transgressive segregation (Rieseberg et al. 1999).

A particular form of transgressive segregation may underlie the final scenario that has the potential to maintain steep clines in mtDNA. The existence of mitonuclear incompatibilities between two hybridizing taxa can result in decreases in fitness in hybrids due to failure of mitochondrial processes in individuals carrying incompatible mitochondrial and nuclear genotypes. This is an example of endogenous (genome dependent) selection. These hybrid incompatibilities would tend to reduce introgression of the mitochondrial genome of one parental species into the nuclear background of the other species, resulting in a steep cline in mtDNA. Steep clines in nuclear loci would also be predicted to occur in nuclear-encoded genes that produce products that directly interact with mitochondrially encoded gene products, since these are the genes that are most likely to produce incompatibilities in hybrids. In this scenario, the parental taxa may or may not differ in mitochondrial function but mitochondrial dysfunction (i.e., transgressive segregation resulting in suboptimal mitochondrial function relative to either parental taxon) would be predicted in hybrids.

Note that these three scenarios are not mutually exclusive. For example, local adaptation of mitochondrial processes or mitonuclear incompatibilities could arise in a system that has undergone divergence in allopatry followed by recent secondary contact, or could occur in the context of a range expansion. In addition, local adaptation of mitochondrial function could increase the probability of mitonuclear incompatibilities arising, resulting in a coupling between exogenous selection due to local adaptation and endogenous selection as a result of genomic incompatibilities in hybrids (Bierne et al. 2011).

With these complexities in mind, it is clear that an integrated examination of patterns of genetic variation in both the mitochondrial genome and the nuclear genome and of the functional consequences of mitonuclear mismatch on mitochondrial processes is required in order to evaluate the predictions of the mitochondrial species concept. Currently, there are only a few species for which these types of integrated examinations have been undertaken (Barreto and Burton 2013; Wolff et al. 2016; Baris et al. 2017; Barreto et al. 2018). Here we use one such species, the Atlantic killifish *Fundulus heteroclitus*, which provides an interesting case study in which to test the predictions of the mitonuclear species concept.

Killifish: a case study in mitonuclear ecophysiology

Populations of Atlantic killifish are found along the Atlantic coast of North America from Newfoundland (Dickinson 1974) to northern Florida (Gonzalez et al. 2009). There is a steep cline in mitochondrial genotypes among killifish populations along this coast (Gonzalez-Vilasenor and Powers 1990; Strand et al. 2012; McKenzie et al. 2016; Healy et al. 2018) with the mitochondrial genome transitioning from a characteristic northern haplotype to a characteristic southern haplotype in central New Jersey. There is also a steep thermal cline along this coast, such that northern populations of killifish experience temperatures that are, on average, about 10°C cooler than do southern populations of killifish, which has the potential to drive local thermal adaptation (Schulte 2007, 2014). Some populations of killifish also exhibit exceptionally high allozyme polymorphism, and there are clines in multiple allozymes along the coast (Place and Powers 1978; Powers and Place 1978). For these reasons killifish have been used as a model for population genetic studies and studies of environmental adaptation for many decades (Place and Powers 1978; Ropson et al. 1990; DiMichele et al. 1991; Powers et al. 1991; Powers and Schulte 1998; Burnett et al. 2007). These previous studies provide a

wealth of both population genetic and functional data with which to examine the relative roles of neutral demographic processes, local adaptation to the environment, and mitonuclear interactions in shaping and maintaining this steep mitochondrial cline, and thus make this species an ideal test case for the principles underlying the mitonuclear species concept.

The role of demography

There is substantial evidence to suggest that demographic effects have played an important role in shaping patterns of genetic variation in killifish (Adams et al. 2006; Duvernell et al. 2008). During the last glaciation, much of the current habitat of killifish bearing the northern mitochondrial haplotype was covered by the Laurentide ice sheet, which reached as far south as northern New Jersey (Mickelson and Colgan 2003), very close to the current contact zone between the northern and southern killifish mitochondrial types. This suggests that, at least for northern killifish, there is likely to have been a strong impact of Pleistocene glaciation on phylogeographic patterns of genetic variation. Examination of patterns of genetic variation in the mitochondrial genome and at nuclear microsatellites suggest that populations of killifish bearing the northern mtDNA type may have persisted in one or more northern refugia and then expanded their range both northwards and south into New Jersey as the glaciers retreated (Adams et al. 2006; Haney et al. 2009). With the glacial retreat, the Hudson River was a main channel for meltwater, and this region of high flow could have presented a substantial barrier to migration and gene flow between the northern and southern types of killifish until the final retreat of the glaciers.

Consistent with the hypothesis that secondary contact between a previously isolated northern and southern form has shaped patterns of genetic variation in killifish, there are many nuclear loci that display clinal patterns along the Atlantic coast in this species (Strand et al. 2012; McKenzie et al. 2016). There are also replicate clines in both mitochondrial and nuclear genes up the rivers in the Chesapeake and Delaware Bay systems (Smith et al. 1998), with northern genotypes being present in the upper, freshwater, portions of these rivers and southern genotypes being present at the coastal mouths. In addition to these genetic patterns, there are also consistent differences in egg and adult morphology, and in a variety of physiological traits (Morin and Able 1983; Able and Felley 1986; Scott et al. 2004; Fangue et al. 2006, 2008; Whitehead et al. 2011) along the coast and in the rivers of the Chesapeake Bay, which is also consistent with a hypothesis of secondary contact between differentiated northern and southern forms. Indeed, these patterns have been sufficient to allow the recognition of two subspecies of *F. heteroclitus*, which have been designated *F. heteroclitus macrolepidotus* in the northern part of the range, and *F. heteroclitus heteroclitus* in the southern part of the range (Jordan and Everman 1896; Morin and Able 1983).

Evidence of environmental adaptation

Despite the clear role for secondary contact in shaping patterns of genetic variation in killifish, the cline in mtDNA haplotype is the steepest among those that have been assessed to date (Strand et al. 2012). Although, as discussed previously, unusually steep clines in mtDNA can be produced by strictly neutral processes including sex-biased gene flow and differences in effective population size between the nuclear and mitochondrial genomes (Toews and Brelsford 2012), it is also possible that selection could be acting to shape this cline. The northern and southern subspecies of killifish differ substantially in a variety of whole-organism traits, including metabolic rate (Fangue et al. 2009; Brennan et al. 2016, 2018), developmental rate (DiMichele and Westerman 1997; McKenzie et al. 2017), upper and lower thermal tolerance (Fangue et al. 2006; Healy et al. 2018), hypoxia tolerance (McBryan et al. 2016; Healy et al. 2018), and salinity tolerance (Scott et al. 2004; Whitehead et al. 2011; Brennan et al. 2018). Taken together, the differences in whole-organism traits between the subspecies are consistent with local adaptation of northern killifish to conditions associated with northern refugia at glacial margins (where temperature and salinity would be low), relative to southern killifish that may be adapted to warmer, more saline, and more hypoxic water (Schulte et al. 2018).

For local adaptation to have influenced the shape of the mtDNA cline there must be functional differences between the subspecies at the level of the mitochondrion. However, it is critical to note that simply observing differences in mitochondrial function between northern and southern killifish is not sufficient to establish that the mitochondrial genome has been the target of this apparent local adaptation, because these comparisons involve taxa that have undergone simultaneous differentiation of both nuclear and mitochondrial genes. On the other hand, in the absence of any functional differentiation, it is

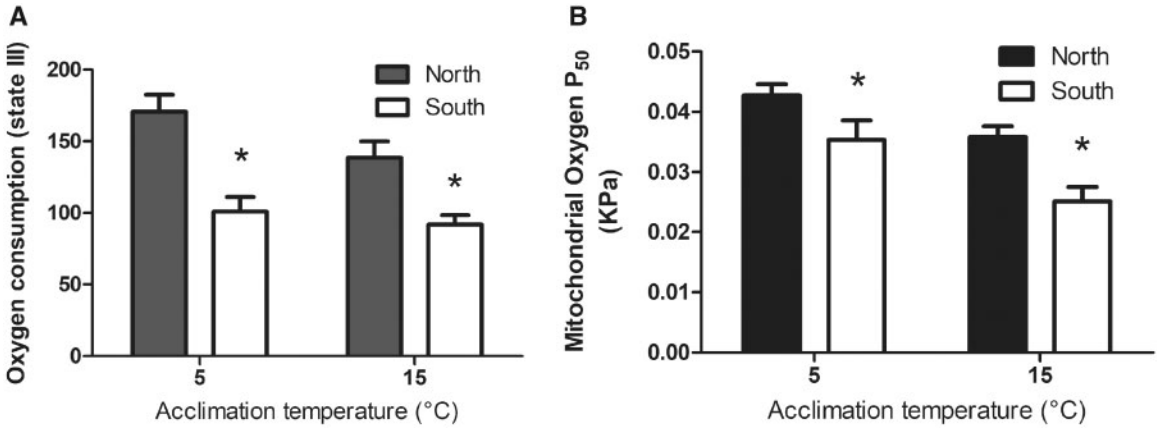

Fig. 1 Functional differences in liver mitochondria between northern and southern killifish. (**A**) Respiratory capacity is greater in northern killifish (filled bars) compared to southern killifish (open bars) at two acclimation temperatures. State III (ADP phosphorylating) respiration was assessed in isolated mitochondria provided with a mixture of saturating substrates (glutamate, pyruvate, malate, succinate) and ADP. Respiration is expressed per mg of mitochondrial protein (Chung et al. 2018). (**B**) Mitochondrial oxygen binding affinity is greater in liver mitochondria from southern killifish (open bars). The P_{50} for oxygen is the oxygen partial pressure at which mitochondrial activity is half maximal. High P_{50} indicates low oxygen affinity (Chung et al. 2017b).

unlikely that the mitochondrial genome has been targeted by exogenous natural selection.

There is ample evidence that there are functional differences between the mitochondria of northern and southern killifish. For example, liver mitochondria from the northern subspecies have greater functional capacity per unit mitochondrion than do liver mitochondria from the southern subspecies (Fig. 1A), and this difference persists regardless of acclimation temperature (Chung et al. 2018). These differences can be observed at many of the respiratory complexes of the ETS, including complexes I, II, and IV, and are also apparent when mitochondria are tested using different substrates (Chung et al. 2017b; Chung et al. 2018). Proton leak through the membrane also differs, suggesting that there may be differences in mitochondrial efficiency between the subspecies (Bryant et al. 2018; Chung et al. 2018). In liver mitochondria there are also clear differences in mitochondrial membrane lipid composition between the northern and southern subspecies, including differences in the amount and type of cardiolipin (Chung et al. 2018), which is a lipid that is known to affect the functional properties of mitochondria (Hoch 1992; Haines and Dencher 2002; Paradies et al. 2014; Mejia and Hatch 2016). Liver mitochondria from northern and southern killifish also differ in their affinity for oxygen (Fig. 1B), with southern fish having higher affinity (lower P_{50}), consistent with their greater whole-organism hypoxia tolerance (Chung et al. 2017b). There are also differences between the subspecies in cold-induced mitochondrial plasticity, with the northern subspecies increasing mitochondrial density and functional capacity in the cold, while the southern subspecies does not (Dhillon and Schulte 2011; Bryant et al. 2018). These differences in liver mitochondrial function are consistent with the observation of countergradient variation in whole-organism metabolic traits in killifish, and suggest that northern killifish perform best under the lower temperatures and shorter growing seasons characteristic of the northern part of the species range (Schulte et al. 2018).

Although there are very clear differences in liver mitochondrial function between northern and southern killifish, many of the differences in mitochondrial function between killifish subspecies are tissue specific. For example, although northern killifish have higher mitochondrial capacity than do southern killifish in liver, we have been unable to detect differences in mitochondrial function in brain or heart (Chung et al. 2017a). However, Baris et al. (2016) found that cardiac mitochondria from southern fish tended to have higher capacity than those from northern fish at lower acclimation temperatures (Baris et al. 2016b), which is the opposite pattern of that observed in liver. Having greater cardiac mitochondrial capacity has been suggested to confer greater thermal tolerance (Iftikar and Hickey 2013), which is consistent with the greater thermal tolerance of southern killifish (Fangue et al. 2006).

Evidence of functional differentiation in mitochondrial properties between northern and southern killifish does not necessarily mean that these differences arose as a result of local adaptation (Garland and Adolph 1994). Nor does it necessarily imply that these differences are the result of adaptation at the mitochondrial, rather than the nuclear, genome as

killifish populations at the extreme of the species range are differentiated at both nuclear and mitochondrial loci. Instead, evidence to assess the potential for a role of adaptive evolution in shaping mitochondrial differentiation can be obtained from analysis of patterns of genetic variation at the mitochondrial genome within and among populations (Barrett and Hoekstra 2011). There is little evidence of diversifying selection operating on the protein coding genes of the mitochondrial genome in killifish (Whitehead 2009), but to date population genomic studies of mtDNA in killifish have used limited sample sizes, and population genomic tests of selection lack power under these circumstances. These tests can also fail to detect selection when selection coefficients are small or when selection influences many loci of small effect. In addition, selection may also act on the non-coding portions of the mitochondrial genome, which have not yet been carefully analyzed. Thus, the lack of evidence for strong diversifying selection on the protein coding components of the mitochondrial genome from the analyses that have been conducted thus far does not necessarily allow us exclude diversifying selection as a process establishing or maintaining the observed steep cline in mtDNA. This is a particularly critical point because simulation studies have suggested that even very weak selection can produce steep clines in mtDNA along an environmental gradient (Irwin 2012). Weak selection is particularly effective when population sizes are large (Lanfear et al. 2014), as is the case in killifish (Sweeney et al. 1998).

The hypothesis that selection may have acted on the mitochondrial genome, despite the current lack of population genomic evidence of selection on this genome, is supported by functional analyses of cardiac mitochondria in fish from a geographically intermediate population that is segregating for both northern and southern mitochondrial genotypes (Baris et al. 2016a). In this study, there was a clear effect of mitochondrial genotype on cardiac mitochondrial function, with fish carrying the southern mitochondrial genotype generally exhibiting greater mitochondrial oxygen consumption than those carrying the northern mitochondrial genotype. However, these relationships were complex, and depended on both acclimation temperature and assay temperature (Baris et al. 2016a). This is consistent with the functional pattern detected between northern and southern populations in cardiac mitochondria (Baris et al. 2016b), and suggests that genetic differentiation at the mitochondrion has functional consequences. Although these functional differences could have arisen via genetic drift, the alternative hypothesis that they are selectively important is supported by the wealth of evidence of functional differentiation in mitochondrial properties in this species.

In contrast to the current lack of population genomic evidence for diversifying selection on the mitochondrial genome, diversifying selection has been an important force shaping genetic variation in the nuclear genome of killifish, and some of this variation is associated with genes of known mitochondrial function (Reid et al. 2016; Brennan et al. 2018; Healy et al. 2018). For example, among the many single nucleotide polymorphism (SNPs) with evidence of selection in the contact zone along the coast, there are two that are close to the genes involved in mitochondrial processes (Healy et al. 2018): the gene coding for isocitrate dehydrogenase, which is a component of the citric acid cycle, and the gene coding for nicotinamide adenine dinucleotide phosphate transhydrogenase, which participates in the maintenance of mitochondrial redox state (Rydström 2006). Similarly, in the Chesapeake Bay, a small subset of the SNPs with evidence of selection are associated with genes involved in mitochondrial processes (Brennan et al. 2018), including important pathways such as the ETS (NADH ubiquinone oxidoreductase, a complex I subunit, and the Rieske subunit of complex III), transport of substances into the mitochondrion (the mitochondrial glutamate carrier), the conversion of pyruvate to acetyl CoA for entry into the citric acid cycle (pyruvate dehydrogenase 3), the regulation of mitochondrial respiration (Sirtuin 3), and mitochondrial translation (a phenylalanine tRNA ligase and several ribosomal proteins associated with the 28S ribosome of the mitochondrion).

In these two contact zones, we have also performed genome-wide association studies to identify SNPs that may be associated with variation in whole-organism traits such as metabolic rate and hypoxia tolerance that could be due to differences in mitochondrial function (Brennan et al. 2018; Healy et al. 2018). Among the SNPs associated with these whole-organism traits are a few that are within genes involved in mitochondrial processes, including MAX-like protein, which is a member of the signaling pathway that regulates mitochondrial biogenesis in response to hypoxia (Schönenberger and Kovacs 2015). Together, these data indicate that there is evidence of selection acting on the nuclear genome of killifish, some of which may involve changes in genes involved in mitochondrial processes. In combination with evidence of functional differentiation at the level of the mitochondria, this suggests that mitochondrial processes may have diverged between the

northern and southern killifish as a result of local adaptation, despite an apparent signature of purifying selection in the mitochondrial genome itself.

This evidence for a role of functionally important (and potentially adaptive) divergence in the nuclear genome with less evidence for adaptive divergence in the mitochondrial genome can be illustrated by the observed differences in mitochondrial oxygen affinity between the two subspecies (Fig. 1B). Oxygen binds to Complex IV of the ETS, which is a multi-subunit enzyme with ~19 core subunits, and the three that form the catalytic core are mitochondrially encoded (Mansilla et al. 2018). There are no fixed differences between northern and southern killifish in the mitochondrially-encoded subunits of complex IV (Whitehead 2009), suggesting that the observed differences in mitochondrial oxygen affinity are likely to be encoded in the nuclear genome. Consistent with the lack of fixed differences in the mitochondrially-encoded subunits of Complex IV, there is no effect of mitochondrial genotype on hypoxia tolerance in hybrid populations of killifish (Flight et al. 2011). Similarly, analyses of cardiac mitochondrial function in an admixed population do not detect an effect of mitochondrial genotype on Complex IV activity (Baris et al. 2016a). Taken together, these data suggest that nuclear-encoded mitochondrial genes may have diverged as a result of adaptive processes, resulting in differences in mitochondrial function at Complex IV, but this is not necessarily associated with divergence in the mitochondrial genome.

Evidence of mitonuclear incompatibility

From the discussion above, it is clear that both historical demography and local adaptation have shaped patterns of genetic variation in killifish, but less is known about the potential role of mitonuclear incompatibility in this species. In killifish there are five fixed differences in the amino acid sequences of mitochondrially encoded genes. These are found in subunits of complex I (two in ND1 and one in ND2 and ND5) and in Complex V (the ATP synthase; in ATPase 8) of the ETS (Whitehead 2009). Both of these complexes contain both nuclear encoded and mitochondrially encoded subunits, and are thus strong candidates for causes of mitonuclear incompatibility. Additionally, there are many fixed differences in the non-protein coding regions of the mitochondrial genome in killifish (Bernardi et al. 1993; Whitehead 2009), which could result in mitonuclear incompatibilities in mitochondrial replication, transcription, or translation (Burton et al. 2013).

If mitonuclear incompatibilities are present in killifish, then it should be possible to observe their effects in populations where the two subspecies come into contact, as in the hybrid zones along the Atlantic coast and up the Chesapeake Bay. These effects should be manifested as barriers to gene flow in these admixed populations and should result in greater reductions in gene flow at the mitochondrial genome and in nuclear encoded mitochondrial genes. Hybrid index in fish from the coastal contact zone shows a strikingly bimodal pattern (Fig. 2), which suggests a deficit of hybrid individuals and a barrier to gene flow between the diverged types. These patterns are evident when hybrid index is estimated either using nuclear microsatellites (Fig. 3A) or with a subset of five nuclear SNPs of a panel of 30 (Fig. 2B) that exhibit clines that are coincident and concordant with the mtDNA cline (McKenzie et al. 2015, 2016, 2017). Among the five nuclear SNPs with clines similar to that of mtDNA are two nuclear-encoded mitochondrial proteins, one of which, the 40S ribosomal protein S17, has a direct interaction with mitochondrially encoded rRNAs. The other nuclear-encoded mitochondrial protein in this dataset, SLC25A3, encodes a mitochondrial phosphate carrier that plays a critical role in allowing oxidative phosphorylation to proceed. However, this protein is not known to have any direct interactions with mitochondrially-encoded gene products, and thus these data provide only circumstantial support implicating mitonuclear incompatibilities in generating the observed barriers to gene flow.

There are also data at the organismal level supporting the potential existence of post-zygotic barriers to gene flow in Atlantic killifish, which is a key prediction of the mitonuclear species concept. When killifish from extreme northern and southern populations are crossed in the laboratory, there are no barriers to fertilization (McKenzie et al. 2017), but post-zygotic breakdown occurs in hybrids. Hatching success is lower for crosses between northern females and southern males compared to all other cross types, and crosses between southern females and northern males have altered developmental rates (McKenzie et al. 2017). These data suggest that there is post-zygotic reproductive isolation between northern and southern Atlantic killifish, although these mechanisms may differ depending on direction of the cross (whether the mother is a northern or a southern fish). Together, these data are consistent with the predictions of the mitonuclear species

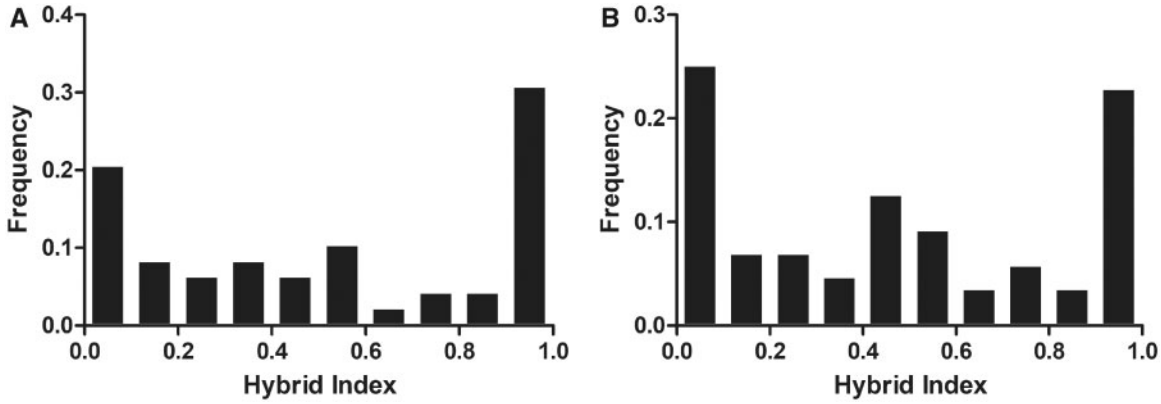

Fig. 2 Bimodal pattern of hybrid indices calculated using (**A**) Nine nuclear microsatellite loci or (**B**) Five nuclear SNPs that exhibit clines coincident and concordant with the mitochondrial DNA cline (McKenzie et al. 2016).

concept, but could also be caused by Bateson–Dobzhansky–Muller incompatibilities involving only nuclear genes. In general, it is critical to bear in mind that crosses between individuals from the geographic extremes of a species range (in this case, between two differentiated subspecies) can fail for a variety of reasons, and thus these experiments provide only a partial test of the mitonuclear species concept.

The clearest prediction that would distinguish mitonuclear incompatibilities from the other possible causes of steep mtDNA clines is that nuclear-encoded mitochondrial genes that are directly involved in mitonuclear interactions should show genetic associations with the mitochondrial genome, resulting in steep clines at these loci and strong evidence of mitonuclear epistasis, while other loci not involved in mitonuclear interactions should show greater introgression across the hybrid zone, and no evidence of epistasis. To test this possibility, we used a dataset of >70,000 nuclear SNPs generated in both the coastal and Chesapeake Bay killifish contact zones in populations that are segregating for both the northern and southern mitochondrial types (Brennan et al. 2018; Healy et al. 2018). We used principle component analysis to determine whether there was evidence of population subdivision based on mtDNA when all nuclear SNPs were analyzed, which would indicate a strong signal of mitonuclear epistasis across the genome. This analysis did not detect any evidence of population subdivision (data not shown), suggesting that there is not a strong signal of mitonuclear epistasis in this species. However, the mitonuclear species concept does not necessarily predict that these signals will be present throughout the genome. Instead, it makes the specific prediction that there should be strong signals of mitonuclear epistasis at or near nuclear genes that perform functions associated with mitochondrial processes, and in particular in those genes that directly interact with mitochondrially encoded gene products. We therefore identified 181 mitochondrially interacting nuclear genes in the killifish genome using homology searching based on known human genes of this type (Zaidi and Makova 2019). For 42 of these genes, our SNP dataset included SNPs within the gene (either in coding regions or introns), and for a further 139 genes there was a SNP located within 27 Kb of the gene. We chose this distance as a cutoff for SNPs that could act as markers as this represents the average size of linkage blocks in killifish (Brennan et al. 2018). This resulted in a dataset that allowed us to assess the extent of cytonuclear disequilibrium for 181 nuclear encoded proteins for which data in other organisms suggests that they have functional interactions with mitochondrially-encoded genes. When only the SNPs associated with these 181 candidate nuclear-mitochondrial interacting genes were analyzed, we found no evidence of population subdivision based on mitochondrial genotype, even when only the 42 genes with SNPs in our dataset that were located within these gene were analyzed (Fig. 3A, B). This indicates that if mitonuclear incompatibilities are important in killifish, they are not occurring broadly across all nuclear–mitochondrial interactions. However, since our SNP database was generated using a reduced representation approach, only a subset of all possible nuclear encoded-mitochondrial genes has been tested, so it remains possible that there are other genomic regions, as yet unexamined, that exhibit such signals. In addition, it is also possible that a mitonuclear incompatibility acting at a single nuclear locus could be sufficient to result in reduced gene flow between fish with alternative mitochondrial genotypes, and this signal could be obscured by

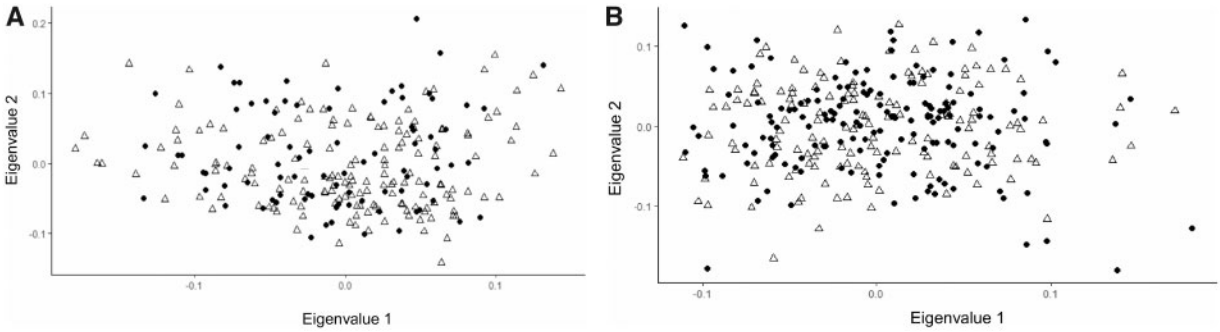

Fig. 3 Population genomic analyses of killifish from admixed populations. Principle component analysis detects no evidence of population subdivision based on mitochondrial genotype in either coastal (**A**) or Chesapeake Bay (**B**) admixed populations when a dataset composed of SNPs located in or near 42 genes that are likely to be involved in mitonuclear incompatibilities is analyzed. Individuals with northern mitochondrial genotype are indicated by filled circles. Individuals with southern mitochondrial genotype are indicated by open triangles.

grouping all potential loci that could be involved in mitonuclear interactions. To test the possibility that there are strong mitonuclear interactions affecting a few key genes, we examined the dataset of 181 SNPs associated with nuclear-encoded mitochondrial genes on a SNP by SNP basis to determine whether any one SNP exhibited evidence of mitonuclear disequilibrium. We found no evidence of disequilibrium between mitochondrial genotype and nuclear genotype at these sites, which suggests that mitonuclear epistasis, if present, is weak, or is acting at sites not examined in this analysis.

As an alternative approach to attempt to detect SNPs that demonstrate mitonuclear epistasis, Baris et al. (2017) used data from a nearby admixed population of killifish along the Atlantic coast that also contains both mtDNA types. They divided the data from this admixed population into two hypothetical sub-populations based on mitochondrial genotype and asked whether there were any nuclear SNPs that were significant F_{st} outliers (which could be indicative of mitonuclear disequilibrium). This analysis detected 349 F_{st} outlier SNPs between the mitochondrial types. None of these SNPs were in ETS complexes, while two were in a mitochondrial ribosomal protein, one was in mitochondrial malic enzyme and one was in acyl-coenzyme A thioesterase 9, an enzyme involved in fatty acid metabolism. We replicated this analysis in our admixed coastal population (Healy et al. 2018) and Chesapeake Bay population (Brennan et al. 2018) using the *fdist* F_{st}-outlier method in the software Lositan, with the complete database of ~70,000 SNPs in these populations (Lopes et al. 2008). In the Chesapeake contact zone, we identified 176 outlier SNPs between mitotypes. Two of these SNPs were located in nuclear-encoded mitochondrial genes: the 30kD subunit of ETS Complex I and in a Phe tRNA ligase. Similarly, in the coastal contact zone, we identified 47 outlier SNPs that separate fish in two groups by mitotypes. Two of these were associated with nuclear-encoded mitochondrial genes: one near Cytochrome c oxidase (ETS Complex IV) subunit 5B and one in acetyl-CoA acyltransferase, which catalyzes the final step of fatty acid oxidation. These analyses implicate different gene products in mitonuclear disequilibrium in different populations, and few of the identified genes have obvious functional interactions with divergent sites in the mitochondrial genome. In addition, these analyses should be regarded with extreme caution, as statistical tests of F_{st} outlier loci can be plagued by false positives (Narum and Hess 2011). To attempt to assess the extent of this problem, we permuted the data for our coastal and Chesapeake contact zone populations by randomly assigning a mitochondrial genotype to each individual while holding nuclear genotype constant. Similar numbers of outlier loci were detected in these permuted datasets, which highlights the potential for generating false positive genetic associations with this approach. Thus, although the identified genes represent interesting candidates for potential mitonuclear incompatibilities, this hypothesis must be rigorously tested. This highlights the critical need for integrated analyses that utilize both population genomic and functional data to attempt to detect mitonuclear epistasis, particularly in systems such as killifish that do not harbor strong genomic signals of mitonuclear incompatibility.

There is some functional evidence of epistatic interactions between the nuclear and mitochondrial genomes in killifish. For example, individuals from an admixed population with a northern mitochondrial genotype but predominantly southern nuclear

background show hypoxia-induced induction of a nuclear encoded mitochondrial elongation factor (EF-Tsmt), whereas those with matched southern nuclear and mitochondrial genotypes do not (Flight et al. 2011). Further studies of mitochondrial capacity and regulation in admixed fish are needed to fully assess the functional basis of this effect. There is also some evidence for an effect of nuclear and mitochondrial genotype on mitochondrial function in an admixed population (Baris et al. 2017). Cardiac mitochondrial function is significantly greater in individuals with a southern mitochondrial genotype and a southern nuclear background at the 349 F*st* outlier SNPs that differed between individuals bearing northern and southern mitochondrial genotypes in a coastal admixed population (Baris et al. 2017), and this difference is in the direction expected based on the observed differences in mitochondrial function between the northern and southern subspecies. However, variation in admixture proportion only accounted for eight percent of the variation in cardiac mitochondrial function, suggesting that the effects of variation at these nuclear alleles on mitochondrial function is relatively modest. In addition, there was no clear evidence for strong negative epistatic effects causing transgressive segregation, as individuals with mismatched nuclear and mitochondrial genotypes had intermediate mitochondrial function, rather than showing evidence of mitochondrial dysfunction that might indicate strong mitonuclear incompatibilities. However, it is possible that mitochondria with intermediate functional properties convey a fitness disadvantage in the environmental context of the hybrid zone, and thus coupling between exogenous and endogenous selection could be playing a role in this system. Similarly, it is possible that the negative epistatic interactions between nuclear and mitochondrial alleles could manifest in aspects of mitochondrial function that were not assessed in these studies, such as reactive oxygen species production, rather than in respiratory rates. In addition, it is important to note that these experiments were performed using wild-caught adult fish from the hybrid zone, and it is possible that individuals harboring strong mitonuclear incompatibilities may be eliminated by selection during early development or at juvenile stages, leaving only those individuals with reasonably functional mitochondria in the adult population. Overall, it is clear that additional functional data across life stages and in multiple tissues will be required to determine whether mitonuclear incompatibilities are an important force shaping genetic variation in killifish.

Conclusions

The results from the studies reviewed here suggest that multiple processes have contributed to establishing and maintaining the observed steep cline in mitochondrial genotype in Atlantic killifish, including historical demography, local adaptation, and possibly mitonuclear incompatibilities. In general, the available evidence suggests that selection has acted to shape mitochondrial function in this system, but this selection may be relatively weak and may involve multiple loci each of small effect. This pattern is consistent with the accumulating evidence for polygenic adaptation in this species (Brennan et al. 2018; Healy et al. 2018; Dayan et al. 2019). However, even weak selection is capable of producing sharp disjunctions in mitochondrial genotype (Irwin 2012), and thus may be important in shaping genetic variation in this species. Detecting these weak effects against the strong background resulting from the history of secondary contact in this species or other neutral processes is likely to be a challenge. In contrast to the evidence for local adaptation in mitochondrial function in this species, the evidence for the existence of strong mitonuclear incompatibilities is largely circumstantial. There is no evidence of a strong genomic signature of mitonuclear epistasis, and only limited evidence of hybrid breakdown, which has not yet been tied to mitonuclear interactions. However, as is the case for local adaptation in mitochondrial function, even weak mitochondrial incompatibilities have the potential to result in steep clines in the mitochondrial genome, and thus a possible role for mitonuclear incompatibilities cannot be ruled out. These data provide a strong challenge to the assumption of the mitonuclear species concept that mitonuclear incompatibilities between hybridizing taxa are strong and pervasive, and form a key isolating barrier in the early stages of speciation. Instead, these data suggest a more nuanced interpretation that allows for the possible role of weak mitonuclear incompatibilities and the role of polygenic adaptation. More broadly, these findings emphasize the need for integrative studies that combine population genomics and functional studies of mitochondrial performance to disentangle these interacting effects. Such studies are needed across multiple species to determine whether the mitonuclear compatibility species concept, as currently formulated, has broad applicability.

Funding

This work was supported by an NSERC (Natural Sciences and Engineering Research Council of

Canada) Discovery Grant to P.M.S., NSERC graduate scholarships to T.M.H., D.J.C., and H.J.B., a Michael Smith Travelling Fellowship to D.J.C., and funding from the National Science Foundation (DEB-1265282 to A.W., and DEB-1601076 to R.S.B. and A.W.). The Society for Integrative and Comparative Biology (SICB) provided support for P.M.S. to participate in the symposium: Beyond the Powerhouse: Integrating Mitonuclear Evolution, Physiology, and Theory in Comparative Biology.

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