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Genetic Architecture of Physiological Phenotypes: Empirical Evidence for Coadapted Gene Complexes[1]

RONALD S. BURTON,[2] PAUL D. RAWSON,[3] AND SUZANNE EDMANDS[4]

Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093-0202

SYNOPSIS. **Physiological phenotypes are the result of the coordinated function of many genes, some of which may be differentiated between conspecific populations. Within any one population, natural selection will favor evolution of a coadapted set of alleles which optimizes physiological performance and reproductive success. The existence of such coadapted gene complexes may be assessed by assaying phenotypes of interpopulation hybrids: inferior performance of hybrids suggests that the allelic combinations present in the parental populations are coadapted. This approach has been used to examine the genetic architecture of physiological traits in the copepod *Tigriopus californicus,* a species characterized by sharp genetic differentiation of populations. Developmental time and response to osmotic stress both show pronounced F_2 hybrid breakdown, a result consistent with genetic coadaptation within populations. To better understand the biochemical and molecular mechanisms underlying hybrid breakdown, we are investigating a specific biochemical phenotype, the activity of the enzyme cytochrome c oxidase (COX). COX (encoded by multiple nuclear and mitochondrial genes) catalyzes the oxidation of cytochrome c (encoded by a nuclear gene). Two approaches are being used to address the extent of coadaptation (both among nuclear genes and between nuclear and mitochondrial genes) underlying COX function: (1) studies of the DNA (and inferred amino acid) sequences of component genes among populations in search of coordinate patterns of amino acid substitution across loci, and (2) direct studies of COX function in interpopulation hybrids and backcrosses. These approaches provide evidence for the existence of nuclear/nuclear and/or nuclear/mitochondrial coadaptation within natural populations of *T. californicus.***

INTRODUCTION

Developmental and physiological processes require the complex orchestration of gene expression in space and time. Production of viable phenotypes dictates that deleterious mutations are selected against and that only alleles capable of harmonious interaction will integrate into a population's gene pool (Wright, 1969; Mayr, 1970; Wallace, 1981). By definition, then, the genetic composition of a species might be viewed as a set of coadapted alleles across gene loci that jointly produce a viable, reproducing organism. The unique nature of each species' gene pool is typically evident in the inviability or infertility of interspecific hybrids. It is not unreasonable, then, to view speciation as the process by which new genic interaction systems or coadapted gene complexes are established at the population level. Although this view is perhaps not universally applicable, it is not particularly controversial.

What is more controversial is the extent to which *intraspecific* genetic variation is organized into coadapted gene complexes similar to those so evident at the interspecific level (see Wallace, 1991). Much of the controversy stems from a powerful result derived from theory: in the absence of strong positive epistasis, recombination rapidly decays linkage disequilibrium thereby preventing stable allelic associations except among the most tightly linked loci. How-

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[2] E-mail: rburton@ucsd.edu

[3] Present Address: School of Marine Science, University of Maine, Orono, Maine 04469-5751.

[4] Present Address: Department of Biological Sciences, University of Southern California, Los Angeles, California 90089-0371.

ever, this objection to the maintenance of different coadapted gene complexes within a species implicitly assumes that there is ample gene flow among conspecific populations to insure that all alleles are introduced to all populations. When gene flow is restricted, conspecific populations will diverge genetically via the processes of mutation, natural selection, and genetic drift. Such differentiation will only confer adaptation to local environments when selective forces prevail. However, even when drift prevails, the interactive nature of gene action will result in genomic coadaptation within populations (Wright, 1969; Mayr, 1970; Wallace, 1981). Hence, while population divergence may be stochastic, the overall genetic composition of a population is unlikely to be a random set of the allelic variants extant in the species.

Evidence for this view of differentiation comes from experiments where individuals from geographically isolated populations are mated in the laboratory: reduced fitness (and increased variance in fitness) is commonly observed among the F_2 hybrids (*e.g.*, see review in Endler, 1977). Since the F_2 hybrid individuals are recombinants between the viable individuals present in each of the natural populations, the observed "hybrid breakdown" is indicative of some sort of genetic incompatibility among conspecific populations and may be considered evidence for genomic coadaptation within each population. Despite the fact that this phenomenon was first observed a half-century ago (*e.g.*, see Dobzhansky, 1950, 1970; Hedrick *et al.*, 1978), little progress has been made in understanding the genetic mechanisms underlying hybrid breakdown (Whitlock *et al.*, 1995; Burton, 1990*b*). What genes are involved? Does hybrid breakdown result from failure to regulate gene activities or are the structural gene products themselves unable to function when differentiated gene pools are mixed?

Using the intertidal copepod *Tigriopus californicus* as a model system, we have carried out several studies aimed at understanding F_2 hybrid breakdown at a physiological and biochemical genetic level (Burton, 1986, 1987, 1990*a*, *b*). Natural populations of *T. californicus* show sharp and temporally stable genetic differentiation over relatively short geographic distances (Burton and Feldman, 1981; Burton, 1986, 1997, 1998); unique alleles at high frequencies at enzyme encoding gene loci often distinguish populations occupying habitat patches separated by only a few kilometers of sandy beach; however, there is not a significant relationship between allozyme genetic distance and geographic distance between populations (Burton, 1994). Mitochondrial DNA sequence differentiation is also remarkable: for example, over 17% divergence has been documented in the cytochrome c oxidase subunit I (COX I) gene between *T. californicus* populations located on opposite sides of Santa Cruz Island (Burton, 1998). The patchy distribution of genetic variants does not reflect any obvious differences in local habitats and suggests that much of the differentiation among populations may not directly confer local selective advantage (Burton, 1987). This is, however, difficult to assess because supralittoral rocky habitats inhabited by *T. californicus* experience extensive variation in physical parameters on all temporal time scales (hourly, daily, and seasonally). In any case, as pointed out above, stochastic differentiation does not preclude the evolution of coadapted complexes within populations.

Results of several inter-population hybridization studies indicate that although F_1 hybrids are physiologically similar to their parents (when reared and tested in a common laboratory environment), F_2 inter-population hybrids have reduced survivorship following hyperosmotic stress (Burton, 1986) and increased developmental times compared to parental populations (Burton, 1987, 1990*b*). Although stress survivorship showed some evidence for a regional effect—lowest survivorships were observed when most distant populations were hybridized—this was not the case for other phenotypes. Developmental times for F_2 hybrids between Santa Cruz and La Jolla populations (over 500 km apart), for example, showed significantly less breakdown than did those between La Jolla and Los Angeles populations (less than 150 km apart). Because there is no significant relationship be-

tween allozyme genetic distance and geographic distance between populations, there is no *a priori* reason to expect a relationship between levels of hybrid breakdown and geographic distance separating populations.

Another approach to the analysis of coadaptation is to focus directly on the effects of genetic background on the fitness consequences of specific alleles. Alleles having high fitness in their "home" genetic background may have deleterious effects in the genetic environment of a different population where they have not been subjected to coadaptive evolution. Burton (1987) presented a simple test of this hypothesis. Using allozyme markers to demonstrate the origin of chromosomal segments in F_2 interpopulation hybrids, it was found that viable segments from one natural population could become semi-lethal when homozygous in F_2 hybrids. In the most extreme example, the homozygous viability of a chromosomal segment from La Jolla (marked with a diagnostic malic enzyme allele) showed a relative viability of 70% in La Jolla/Santa Cruz F_2 hybrids and only 7% in La Jolla/Los Angeles F_2 hybrids. Although striking in both cases, it is interesting to note that, again, the magnitude of the effect was not predictable on the basis of the geographic proximity of the populations.

In an attempt to further elucidate the biochemical and genetic mechanisms underlying hybrid breakdown of physiological traits, we have now focused attention on the terminal step of the mitochondrial electron transport chain, the transfer of electrons from cytochrome c to molecular oxygen catalyzed by COX. This system was selected for a diversity of reasons: (1) previous work on population structure in *T. californicus* had revealed extensive genetic divergence in COX I encoded in the mtDNA, (2) a simple and sensitive assay exists for the characterization of COX enzyme activity, (3) previous studies in other systems demonstrated that there is a high degree of species-specificity in the functional interaction of COX and cytochrome c (Osheroff *et al.*, 1983), and (4) at the molecular level, the rates of amino acid replacement for the mitochondrial encoded cytochrome c oxidase subunit II (COX II) and cytochrome c are highly correlated among mammalian lineages (Baba *et al.*, 1981; Cann *et al.*, 1984), suggesting a pattern of nuclear/mitochondrial coadaptation across lineages. The intriguingly complex structure of COX (up to 10 subunits encoded in nucDNA and three subunits encoded in mtDNA), provides the opportunity for investigating the extent of both nuclear/nuclear and nuclear/mitochondrial gene interactions. Finally, despite this complexity, the crystal structure of COX (as well as cytochrome c) has been resolved (Tsukihara *et al.*, 1996, and see review by Capaldi, 1996), so the potential exists to track down the functional consequences of polymorphisms in this system to the level of enzyme/substrate interactions. Although we have yet to take advantage of many potential features of this system, we report here on progress to date in two areas: (1) analysis of patterns of DNA sequence variation at the COX I and the cytochrome c gene loci across California *T. californicus* populations, and (2) analysis of COX enzyme activities in natural populations and interpopulation hybrids.

TIGRIOPUS STUDY SYSTEM AND METHODOLOGIES

This paper presents a synopsis of both completed studies and work currently in progress. In most cases, details of methodology are already published. We provide only a brief overview of features of the study system and general biochemical and molecular methods.

Tigriopus californicus is an abundant harpacticoid copepod inhabiting high intertidal and supralittoral rock pools along the Pacific coast of North America from at least southern Alaska to central Baja California, Mexico (S. Edmands, personal observation). The life cycle of *T. californicus* includes six naupliar and six copepodid stages with the sixth copepodid stage being the adult. Adult females brood egg sacs until they hatch and all subsequent life stages are free swimming. Although the entire life cycle may take place within the confines of a single tide pool, all life stages possess dispersal capacities and large numbers of animals are irregularly scoured from pools during winter storms. Environmental con-

ditions in these pools fluctuate rapidly and reach extreme values of salinity and temperature. Although *Tigriopus* sp. are remarkable in their physiological tolerances, they have no long term resting or dormant stages. Hence, extended periods (days to weeks) of extreme low salinity (<5% seawater) and periods of complete desiccation result in 100% mortality of resident copepods. Although extinction events in individual pools are common (Dybdahl, 1994), the population of *T. californicus* inhabiting the network of pools located on a single rock outcrop shows remarkable genetic stability through time (Burton, 1997).

Most important for analysis of coadaptation is the fact that *T. californicus* is easily cultured in the lab through multiple generations. Adult males clasp immature female copepods until the latter undergo their terminal molt; at that time males inseminate and release the females. This mating system allows convenient manipulation of mates—virgin females are obtained by separating clasped pairs; the females are then placed in petri dishes with adult males (easily recognized by their geniculate antennae). Females only mate once (Burton, 1985), so following insemination, they can be individually isolated in 15 × 100 mm petri dishes where they will produce a series of broods of offspring, all from the same father. Such broods can be allowed to sibmate, establishing isofemale lines. The generation time of *T. californicus* is approximately 24 days at 20°C, permitting analyses of interpopulation crosses to extend to F_1 and F_2 generations (Burton 1986, 1987, 1990*a*, *b*) as well as through multiple generations of backcrossing.

Genetic variation in COX subunits I and II (mtDNA) and the cytochrome c (nucDNA) genes

Our methodologies for studying polymorphism in nuclear and mitochondrial genes largely follow standard protocols for polymerase chain reaction (PCR) amplification of target genes followed by direct sequencing of the PCR products. Although we initially experienced difficulty extracting DNA from single *T. californicus* (wet weight 30 μg) for use as template for PCR (Burton and Lee, 1994), we have found that simply heating single intact adults for 8 minutes at 100°C directly in the PCR tube with 30 μl of deionized water consistently yields adequate template for both mtDNA and nucDNA PCR.

Genetic variability of a 500 bp fragment of the COX I gene has been analyzed by direct DNA sequencing following polymerase chain reaction amplification (Burton and Lee, 1994). A smaller fragment of the gene has now been sequenced from over 100 individual copepods representing 19 different natural populations of *T. californicus* (Burton, 1998, and unpublished data). Because of the great degree of sequence variation among populations for the COX I gene, we have no PCR primers that successfully amplify this gene from all populations. The series of primers employed for different populations are presented in Burton (1998). For the present purposes, we will focus on four populations for which we also have data on the cytochrome c gene: Santa Cruz (SC) and Carmel (CAR) in central California and Los Angeles (AB = Abalone Cove on the Palos Verdes Peninsula) and San Diego (SD) in southern California.

Data on DNA sequence variation in COX II from the SD and SC populations were obtained following design of degenerate primers based on known arthropod COX II sequences. Full details will be published elsewhere (Rawson and Burton, in prep.). Inferred amino acid sequences for both COX subunits assumed the *Drosophila* mtDNA genetic code.

To obtain sequence of the cytochrome c gene, Rawson et al. (submitted) used the degenerate primers of Palumbi and Baker (1994) on a *T. californicus* cDNA library. The short sequence obtained was used to design primers that were then paired with vector primers to amplify the 3′ and 5′ ends of the cDNA. Primers designed from the ends of the gene were then used to amplify the entire gene from 11 individuals representing four natural populations of *T. californicus*. Interestingly, the gene includes an intron (approximately 500 bp long) located in the same position as in vertebrate cytochrome c genes. This intron is absent in insect (*Drosophila*) cytochrome c genes.

Mating				Offspring Genetic Composition (expected average)	
Female Parent	X	Male Parent	Offspring	nucDNA	mtDNA
Population A	X	Population B	F1	50%A : 50%B	100% A
F1	X	Population B	F1BK X	25%A : 75%B	100% A
F1BK X	X	Population B	F2BK X	12.5%A : 87.5%B	100% A
	•				
	•				
	•				
F(N) BK X	X	Population B	F(N+1)BK X	**approx 100% B**	**100% A**

FIG. 1. Crossing scheme employed to effectively move mitochondria to foreign genetic backgrounds. Following F_1 hybridization, females are repeatedly backcrossed to males from the paternal population, resulting in animals with maternally-derived mitochondrial genome and a largely paternally-derived nuclear genome. A control set of crosses involves backcrossing to males from the maternal population, a procedure that returns the maternal mitochondria to its original nuclear background.

COX activity assays

Activity of cytochrome-c oxidase (EC 1.9.3.1) was measured by a standard spectrophotometric end-point assay based on the oxidation of horse heart cytochrome c (Type VI from Sigma Chemical Co., reduced with ascorbic acid) by crude homogenates of *T. californicus* (Simon & Robin, 1971). Individual copepods were homogenized in 200 μl of freshly mixed 50 m*M* NaH_2PO_4 pH 7.1, with 0.05% Tween 80 detergent and centrifuged for 5 min at 2000 g; two 40 μl aliquots of the supernatant were distributed into each of two 96-well microtitre plates for replicated COX activity and total protein measures (used to normalize activities across different size animals). Further details of the methods are in Edmands and Burton (1998).

Manipulation of cytonuclear genotypes

A direct method for testing for interactions between nuclear and cytoplasmic genetic elements (*e.g.*, mitochondrial genes) employs laboratory crosses to substitute mitochondria from one population onto the nuclear background of a second population. We are employing a protocol previously used in *Drosophila* studies (*e.g.*, Kilpatrick and Rand, 1995) to carry out such substitutions. In brief, the method takes advantage of the differences in modes of inheritance of nuclear versus mitochondrial genomes; while the former is biparentally inherited, mtDNA is typically maternally inherited. Hence, an F_1 interpopulation hybrid between a female from population A and a male from population B will have a 50:50 mix of A and B nuclear genes, but only population A mtDNA. If the F_1s are then backcrossed to the paternal line (B), the resulting progeny will still have only A mitochondria, but will have 75% B nuclear genes. Continued backcrossing to the paternal population reduces the expected fraction of A nuclear genes by half each generation (Fig. 1). This expectation, of course, assumes that there is no selection and no paternal contribution of cytoplasm. An elegant feature of this scheme is that there is an obvious control set of crosses: backcrossing to the maternal population should simply put the mtDNA right back on its original nuclear genomic host. Although our intended focus in these experiments concerns interactions between nuclear and mitochondrial genomes, it is important to note that other cytoplasmic factors or maternally inherited parasites might have an effect which we are here attributing to mitochondria only for the sake of simplicity in presentation.

RESULTS

DNA and inferred protein sequences of COX I and cytochrome c

DNA sequence variation in a fragment of the COX I gene (mtDNA) among natural

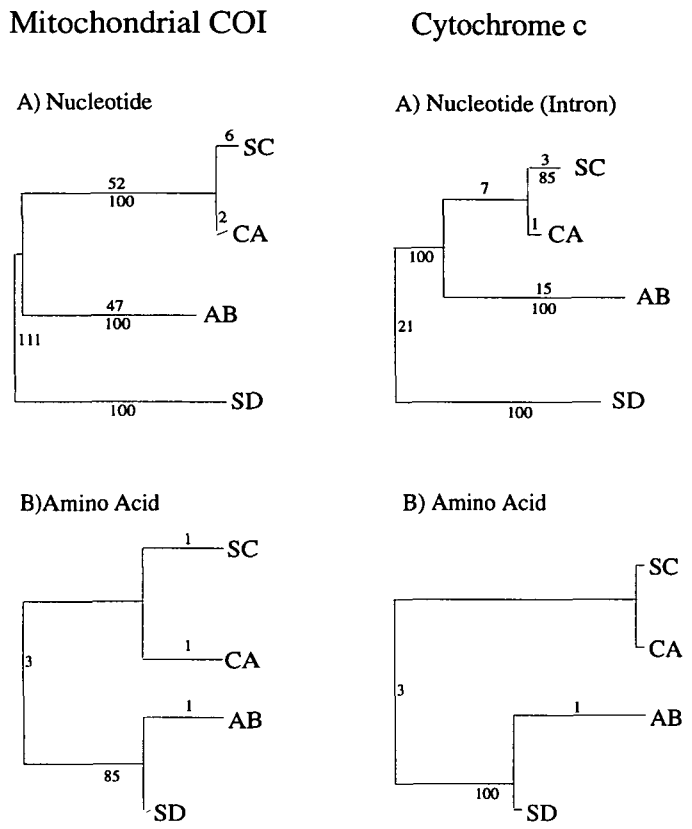

FIG. 2. Maximum parsimony trees showing the relationships among nucleotide (A) and inferred amino acid (B) sequences for COX subunit I and cytochrome c genes in *T. californicus* populations. Numbers above branches are the absolute number of substitutions (within the 543 bp fragment for COX subunit I and 304 bp for the cytochrome c intron); number below branches show level of bootstrap support for the relevant nodes. The amino acid sequences analyzed were 181 and 97 residues long for the two genes respectively.

populations of *T. californicus* has been previously described (Burton and Lee, 1994; Burton, 1998). Within population variation is low so only single sequences for each population are shown in Figure 2. Although most nucleotide substitutions are silent, three diagnostic amino acid substitutions distinguish central from southern California populations (Fig. 2). In considering the functional significance of COX sequence variation, it is important to note that the COX II and COX III genes are also encoded in the mtDNA; hence all three subunits are inherited as a unit. Hence, it is important to determine levels of amino acid substitutions in each of the COX subunits. This work is currently underway. Preliminary COX II sequence data (Rawson and Burton, unpublished) shows that although inferred amino acid sequences do not vary within populations, differentiation between SD and SC populations is substantially higher (>4-fold) than that observed in the COX I fragment, with 18 of 226 residues (approximately 8%) substituted (Fig. 3).

Of the 291 nucleotide positions compared within the cytochrome c coding region, 14 sites were variable, including 10 synonymous sites. However, the presence of an intron within this coding sequence provides a more highly variable marker for analysis of population genetic relationships. The cytochrome c intron is strongly differentiated between the four study populations (Figure 2). More surprisingly, inferred amino acid sequences from the exons differ among the populations; to our knowledge this is the first report of intraspecific poly-

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SD1     MMSWGQLGFQDAGSYFMEEFMYFHDFTMMMMLGVLSYVVVFLILGLTSGW
SD2     ..................................................
SCN1    .S..C.....................................V...L...
SCN2    .S..C.....................................V...L...

SD1     VDSGFVEGQLMEFMWTMFPALMLMQMAFPSLLLLYLMEDFSKSFLVCKVM
SD2     ..................................................
SCN1    ....................I.........................V
SCN2    ....................I.........................V

SD1     GHQWYWSYEMKTESSELLASVDCYMLPREDAFGMRLLLTDEFLMLPMNVP
SD2     ..................................................
SCN1    ................M...........G.....................
SCN2    ................M...........G.....................

SD1     VRMLVTSDDVIHSWTLPSLGIKGDAVPGRLNQLNLTLNRKAMFYGQCSEM
SD2     ..................................................
SCN1    ..........M....I....M.............V..S............
SCN2    ..........M....I....M.............V..S............

SD1     CGANHSFMPMMLMGVYSWEFFAWLDR
SD2     .........................
SCN1    .........V.....T.PQ..M....
SCN2    .........V.....T.PQ..M....
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FIG. 3. Alignment of inferred amino acid sequences from two COX subunit II genes from each of two natural populations.

morphism for this highly conservative gene. Like the COX I sequences, the amino acid sequences of cytochrome c in the SD and AB populations show a level of similarity that is not observed at the nucleotide level; while the intron nucleotide sequences place AB within the central California clade (with high bootstrap reliability), the amino acid sequence of the functional protein is clearly more similar to that found in the SD population (Rawson *et al.*, submitted).

COX activity in isofemale lines, interpopulation hybrids and backcrosses

COX activity in 40 isofemale lines established from three natural populations and raised in a common garden varied significantly both within and among populations (Fig. 4). In several cases, assays were repeated on multiple days and enzyme activity measurements were found to be repeatable. Within populations, COX activity varied by up to 5-fold (0.009–0.047 nmol/min at Santa Cruz) while among populations COX activity varied by more than 11-fold (0.004–0.047 nmol/min). In a data set including 3 additional populations and 18 additional lines, Edmands and Burton (1998) found that almost 50% of the variance is between populations ($P = 0.001$). Although some patterns are apparent (COX activity is highest in Santa Cruz and lowest in Abalone Cove), there is no clear geographic pattern.

To clarify the mode of inheritance of COX activity, Edmands and Burton (1998) conducted a series of crosses among isofemale lines. Reciprocal crosses were used to test the hypothesis that variation in COX activity is maternally inherited since the catalytic core of COX lies within the mtDNA encoded subunits. Although results

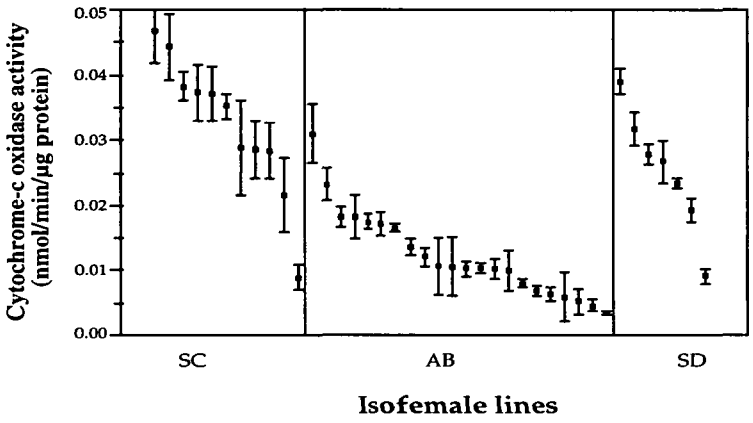

FIG. 4. Cytochrome-c oxidase activity (adjusted for total protein content, gender and sample × plate interaction) for isofemale lines from 3 geographic locations in California listed from north to south SC: Santa Cruz, Santa Cruz County; AB: Abalone Cove, Los Angeles County; SD: San Diego, San Diego County. Each point represents the mean enzyme activity (±SE) for multiple copepods (mean sample size 5.9, range 3–33) from a single isofemale line.

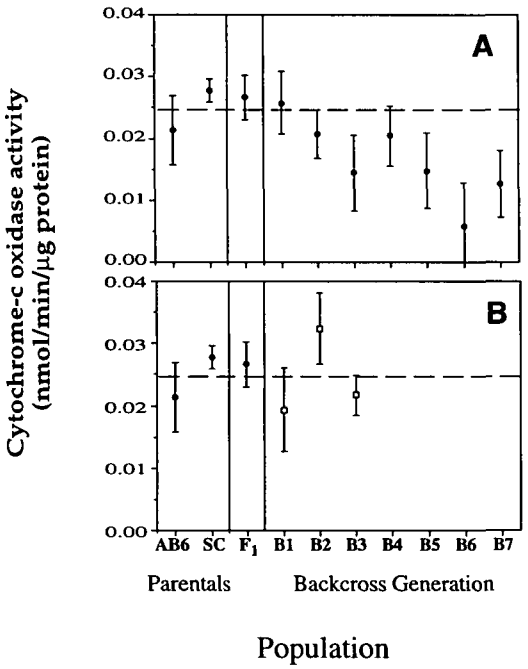

FIG. 5. Cytochrome-c oxidase activities adjusted for total protein content, gender and sample × plate interaction. Each point represents the mean activity (±SE) for a minimum of three copepods. In the experimental cross (panel A), females from isofemale line AB6 were crossed with males from a Santa Cruz (SC) population and hybrid females were repeatly backcrossed to the paternal population. In the control cross (panel B), hybrid females were repeatedly backcrossed to males from the maternal population Abalone Cove (AB). Dashed line denotes the midparent mean.

clearly rejected the maternal inheritance hypothesis, crosses between lines derived from different natural populations showed a consistent pattern of decreased activity in the F_2 generation. This contrasts with the results of intrapopulation crosses (between lines derived from the same population), which showed a strong increase in enzyme activity in the F_1 but no significant drop in activity in the F_2 (Edmands and Burton, 1998).

Most intriguing are the experiments involving repeated backcrosses to introduce mitochondria to foreign nuclear backgrounds. The results of one such experiment are shown in Figure 5. Although COX activity of the AB6 line did not initially differ from that of the SC population, repeated back-crossing appears to have markedly depressed enzyme activity as the AB6 mitochondria are placed on the SC nuclear genetic background (expected proportion of "foreign" nuclear genes exceeds 99% in the seventh backcross generation). Unfortunately the control backcrosses only progressed through three generations (>93% nuclear contribution from the maternal AB6 line) before the populations failed (for unknown reasons); no hybrid breakdown was apparent in COX activity when the mitochondria was returned to its "native" nuclear background.

It is important to note that COX activity standardized to total protein, as it was measured here, is a function of both the amount and activity of the enzyme. The variation in activity observed may therefore be caused by a variety of factors involving either nuclear or mitochondrial genes including mitochondria number, transcription rate, transcript processing, message stability, translation efficiency, protein stability or intrinsic catalytic efficiency (Clark and Wang, 1994).

DISCUSSION

In the absence of extensive gene flow, conspecific populations will differentiate genetically via mutation, selection and genetic drift. Given sufficient time, an expected consequence of this population genetic differentiation is outbreeding depression, the observed reduction of physiological performance or fitness in interpopulation hybrids relative to that found in each natural population (Price and Waser, 1979; Lynch, 1991; Waser and Price, 1994). At least two genetic mechanisms may underlie outbreeding depression (Waser and Price, 1994): (1) if population differentiation is the result of selection and local adaptation, then interpopulation crosses could disrupt these adaptations and hybrids might be ill-adapted to any set of environmental conditions, or (2) where differentiation is the result of stochastic processes, outbreeding depression could result from the disruption of coadapted gene complexes that have evolved in the isolated populations (see Lynch, 1991, for a complete model of the genetic basis of outbreeding depression).

In principle, we may be able to distinguish between the evolution of adaptations to local environments external to the organism and the evolution of coadaptation within an organism's genome. However, although they describe alternate evolutionary processes, adaptation and coadaptation are not mutually exclusive. In fact, the genetic bases of some of evolutionary biology's most elegant local adaptations, such as mimicry in tropical *Papilio* butterflies and the area effects in the snail *Cepaea,* have been found to involve coadapted sets of alleles, often at tightly linked groups of loci or "supergenes" (Wallace, 1981). The tight linkage observed in these supergenes is required to prevent recombination between coadapted sets of alleles, as recombination almost invariably produces poorly adapted phenotypes. Similarly, reduction of recombination within polymorphic inversions of *Drosophila pseudoobscura* are thought to have made possible the evolution of coadapted complexes (Dobzhansky, 1970).

The requirement for tight linkage among loci involved in coadapted gene complexes is relaxed when interpopulation gene flow is restricted. Indeed, population genetic structure which results in nearly independent evolution of conspecific populations will allow coadaptation to span not only entire freely-recombining genomes but also extend to extranuclear genomes (such as mitochondrial DNA). Avise (1991) pointed out that because some enzyme subunits are encoded into the nuclear genome while others are encoded in mtDNA (as is the case of COX), inter-genomic coadaptation may evolve as a consequence genetic divergence between populations. There have, however, been relatively few experimental tests for nuclear/mitochondrial coadaptation. Liepins and Hennen (1977) transplanted single nuclei from *Rana pipiens* blastula cells into enucleated eggs of *Rana palustris* and found that normal COX activity during development requires at least a haploid genomic complement derived from the same species as that from which the mitochondrial genome is derived, a result that is clearly consistent with the concept of an integrated gene pool within a species. In another study, it was found that primate cytochrome c (nuclear encoded) shows a higher reaction rate with primate COX (catalytic subunits are of mitochondrial origin) than with non-primate COX (Osheroff *et al.,* 1983; also see Cann *et al.,* 1984). Perhaps the most elegant intraspecific study is that of King and Attardi (1989), who took human cell lines experimentally depleted of mitochondria and showed that COX activity varied with the source of exogenous mitochondria reintroduced to a common host cell line. Aside from these studies on cytonuclear interactions in the COX system, most experimental studies have focused on

population level measures of fitness interactions between nuclear genome and cytoplasm (*e.g.*, Clark and Lyckegaard, 1988; Kilpatrick and Rand, 1995).

The single most useful feature of the *Tigriopus* system with regard to analysis of the genetic architecture of physiological phenotypes is the remarkable level of genetic differentiation observed among geographic populations. This differentiation has been apparent since our early allozyme studies (Burton and Feldman, 1981), but the extraordinary lengths of time over which *T. californicus* populations remain isolated have only become apparent from DNA sequence data (Burton and Lee, 1994; Burton, 1998). Levels of mtDNA sequence divergence at the COX I locus among *T. californicus* populations are among the highest intraspecific values yet reported, often exceeding 18%. The preliminary data reported here for COX II is even more astonishing, with differentiation between populations at nearly 8% at the amino acid level. With less than 50% of the mtDNA encoded subunits studied (two-thirds of COX I and all of COX III remain), we have already documented over twenty diagnostic amino acid substitutions between the SC and SD populations. It is this unprecedented variation in an enzyme of known physiological importance that has attracted our further investigations. Can such highly divergent mitochondrial genomes function normally when placed on foreign nuclear genetic backgrounds or has there been significant intergenomic coadaptation?

Although an approach focusing on the function of COX in interpopulation hybrids represents the most direct route toward addressing this question, one immediately encounters a technical problem—although we can easily assay COX function with commercially available cytochrome c (typically derived from horse), we might expect the manifestation of coadaptation to be most evident when *T. californicus* cytochrome c is used as substrate. This is particularly true because cytochrome c itself has proven to be differentiated among *T. californicus* populations. While we are currently investigating the possibility of obtaining usable quantities of these cytochrome c variants (either by purification or *in vitro* expression), our functional assays to date have had to employ heterologous cytochrome c. Analysis of coadaptation between COX I and cytochrome c within *T. californicus* populations has, therefore, been limited to the sequence analyses discussed above. The similarity in patterns of population divergence of these two functionally related genes at the amino acid level is suggestive of coadaptive evolution but strong conclusions must await functional analyses.

Edmands and Burton (1998, and Fig. 4) documented extensive variation in COX enzyme activity among isofemale lines derived from several *T. californicus* populations. The mode of inheritance of this variation is clearly complex and likely involves both nuclear/nuclear and nuclear/mitochondrial gene interactions. Intrapopulation crosses showed a strong increase in COX activity in the F_1 and a small and insignificant decrease in activity in the F_2. The increase in the F_1 implies a release from inbreeding depression, which is generally attributed to interactions at nuclear loci (*e.g.*, Charlesworth and Charlesworth, 1987). In contrast to the intrapopulation crosses, interpopulation crosses had a much smaller increase in enzyme activity in the F_1, and a much larger drop in activity in the F_2. The smaller increase in the F_1 implies that any benefits of inbreeding release are opposed by detrimental interactions in hybrids. The strong F_2 breakdown suggests epistatic interactions between loci. Although there is previous evidence for strong nuclear-nuclear epistatic interaction in *T. californicus* (Burton, 1987), nuclear-mitochondrial interactions could also contribute to the observed COX activity patterns in inter-population hybrids. If only a haploid complement of non-foreign nuclear DNA is necessary for normal nuclear-mitochondrial interaction, as was suggested in a study of cytochrome oxidase function by Liepens and Hennen (1977), then nuclear-mitochondrial conflict will not be manifested until the F_2 generation.

A more comprehensive analysis of nuclear/mitochondrial interactions on COX function can be made by moving mitochondria from their home nuclear genetic back-

grounds to foreign backgrounds through a protocol of repeated backcrossing. Edmands and Burton (in prep.) have attempted this approach and obtained the data discussed above (Fig. 5). A pattern of decreasing COX activity as the AB6 mitochondria are moved to a purer and purer SC nuclear background is evident and is exactly what might be predicted by inter-genomic coadaptation. It should be noted that some other sets of interpopulation crosses did not produce any discernible pattern. However, if coadaptation arises during the course of stochastic population differentiation, hybrid breakdown would be expected to be population (if not line) specific and unpredictable.

In the above paragraphs we have reviewed the evidence for the existence of coadapted gene complexes in *T. californicus*, including our previous work involving nuclear/nuclear coadaptation and our more recent evidence of nuclear/mitochondrial intergenomic coadaptation. In total, we think the case is compelling. We now briefly suggest that the results of our analyses have at least three important implications for the study of physiological adaptations:

1) Even conservative physiological phenotypes, including traits such as developmental rates, physiological responses to stress, and regulation of enzyme activities are subject to incessant evolutionary genetic change. Because some evolutionary change results from random chance events (*e.g.*, mutation and drift), the dynamic nature of this process should generally not be viewed as evolutionary "fine-tuning" of adaptations. Such fine tuning undoubtedly occurs, but not all genetic changes within populations contribute to adaptation. Chance events will differ among populations; this will lead to population differentiation of the genetic basis (or architecture) underlying physiological phenotypes to the extent that the populations are genetically isolated.

2) The driving force behind evolutionary change is not always adaptation to the external environment. Subtle genetic changes introduced into a population's gene pool by mutation face constant selective scrutiny favoring the maintenance of harmonious genic interactions. These selective pressures are generated internally, within the genome. Hence, natural selection simultaneously results in both adaptation to the environment and coadaptation within the gene pool. The former process has received much more attention from physiologists than the latter, but both processes can have profound effects on the expression of physiological phenotypes.

3) Analysis of the genetic architecture underlying physiological phenotypes represents an important link between studies of population structure and studies of evolutionary physiology. Assessing the potential importance of coadaptation requires an understanding of the genetic structure of natural populations. In situations where extensive gene flow results in panmixis, allelic interactions on unique genetic backgrounds are likely to be of little importance since each allele will ultimately occur on a homogeneous background facilitated by gene flow and recombination. Only where restricted gene flow results in independently evolving populations will genome-wide coadapted complexes have the opportunity to evolve. Despite decades of allozyme and mtDNA analyses, our understanding of the population genetic structure of most species is poor. Without such understanding, we can have little insight into the importance of intraspecific coadaptation in the evolution of physiological adaptations.

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Corresponding Editor: Todd Glesson