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Chromosomal basis of viability differences in *Tigriopus californicus* interpopulation hybrids

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Abstract

Crosses between populations of *Tigriopus californicus* result in backcross and F_2 hybrid breakdown for a variety of fitness related measures. The magnitude of this hybrid breakdown is correlated with evolutionary divergence. We assessed the chromosomal basis of viability differences in nonrecombinant backcross hybrids using markers mapped to individual chromosomes. To assess effects of evolutionary divergence we crossed one population to three different populations: two distantly related (~18% mitochondrial COI sequence divergence) and one closely related (~1% mitochondrial COI sequence divergence). We found that all three interpopulation crosses resulted in significant deviations from expected Mendelian ratios at a majority of the loci studied. In all but one case, deviations were due to a deficit of parental homozygotes. This pattern implies that populations of *T. californicus* carry a significant genetic load, and that a combination of beneficial dominance and deleterious homozygote–heterozygote interactions significantly affects hybrid viability. Pairwise tests of linkage disequilibrium detected relatively few significant interactions. For the two divergent crosses, effects of individual chromosomes were highly concordant. These two crosses also showed higher heterozygote excess in females than males across the vast majority of chromosomes.

Introduction

Hybridization between divergent populations can result in both increases (heterosis) and decreases (hybrid breakdown or outbreeding depression) in fitness. The genetic mechanisms underlying hybrid fitness are of great interest to both evolutionary and conservation biologists. Evolutionary biologists are interested in what these mechanisms imply about the first stages in the development of reproductive isolation. Conservation biologists are concerned with the risks involved in translocation efforts between declining and stable populations, as well as hybridization between unintentionally introduced and native species or populations.

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First generation (F_1) hybrid progeny often exhibit higher fitness than either parent (heterosis). The genetic basis of heterosis has been studied for nearly a century with little satisfaction in the proposed mechanisms involved. Although more complex models involving epistatic interactions and regulatory mechanisms have recently been proposed, the historically favoured explanation involves either overdominance or the masking of deleterious mutations (Birchler *et al.*, 2003). Some models suggest that even frequency differences of deleterious mutations among populations (a result of genetic drift) can result in heterosis in interpopulation hybrids due to a 'net masking of deleterious effects' (Whitlock *et al.*, 1999).

Reduced fitness can also be exhibited in F_1 hybrids that possess a complete haploid set of each parental genome. The F_1 fitness reductions can be attributed to the disruption of local adaptation, underdominance or epistatic interactions. It is common however, that reductions

in fitness are not manifested until the F_2 or later generations when deleterious interactions between homozygous loci become exposed. It has become widely accepted that the evolution of epistatic incompatibility is explained by the model proposed by Dobzhansky (1937) and Muller (1940). The Dobzhansky–Muller model explains that isolated populations gradually accumulate neutral or advantageous mutations over time. Furthermore, selection for positive epistasis may result in the development of unique coadapted gene complexes within each isolated population. When mating occurs between populations, segregation and recombination can break-up these coadapted gene complexes and bring together mutations that have not been 'tested' together and potentially have harmful effects (Orr, 1996; Turelli *et al.*, 2001).

The intertidal copepod *Tigriopus californicus* is an excellent laboratory system for investigating the genetic consequences of hybridization. This species has a short generation time (23 days at 20°C) and is easily maintained and bred in the lab (Burton, 1987; Edmands, 1999). The genome consists of 12 pairs of chromosomes (Ar-Rushdi, 1963), recombination has not been observed in females (Ar-Rushdi, 1963; Burton *et al.*, 1981), and sex determination is thought to be polygenic with an environmental component (Voorduow & Anholt, 2002a,b). *Tigriopus californicus* inhabits high intertidal pools ranging from Alaska to central Baja California, Mexico. Remarkable genetic differentiation between populations inhabiting different rocky outcrops has been well-documented using allozymes, nuclear and mitochondrial DNA sequence data, and microsatellite loci (Burton & Lee, 1994; Burton, 1998; Edmands, 1999, 2001; Edmands & Harrison, 2003). Despite the extensive genetic divergence observed between populations (mitochondrial COI sequence divergence ranges from 0.2% to 23%), no prezygotic isolation has been observed (Ganz & Burton, 1995; Palmer & Edmands, 2000) and interpopulation crosses in the laboratory produce fertile hybrid offspring (e.g. Burton *et al.*, 1981). Additionally, F_1 offspring typically show slight heterosis while F_2 hybrids show hybrid breakdown in numerous fitness characters including: response to osmotic stress (Burton, 1986); developmental time (Burton, 1987); cytochrome *c* oxidase activity (Edmands & Burton, 1999); hatching number, metamorphosis number and survivorship (Edmands, 1999). Backcross hybrids also show moderate reductions in hatching number, metamorphosis number and survivorship (Edmands, 1999).

Edmands (1999) showed that in F_2 and backcross hybrids, the magnitude of outbreeding depression in a number of fitness characters (hatching number, survivorship number and metamorphosis number) is strongly correlated with evolutionary divergence (COI sequence divergence). In addition, she found that similar levels of outbreeding depression occurred in both recombinant and nonrecombinant backcrosses suggesting that at least some of the negatively interacting loci reside on different chromosomes. Burton (1987) used deviations from Mendelian ratios in allozyme markers mapped to two of 12 *T. californicus* chromosomes to estimate viability effects of each chromosomal region and interactions between the two regions in interpopulation F_2 hybrids. He found in one cross that a parental homozygote genotype was nearly lethal in the hybrid genetic background and that there was significant interaction between chromosomes in another cross.

The study presented here uses the approach of Burton (1987) and expands it to a larger portion of the genome. We use markers mapped to individual chromosomes and three nonrecombinant backcross hybrid lines to assess viability effects of all 12 chromosomes in one cross, 11 chromosomes in a second cross and six in a third cross. While the nonrecombinant backcross approach is relatively coarse, it offers tremendous advantages for initial studies of hybrid breakdown. First, it allows effects of intact chromosomes to be assessed without the complicating effects of recombination. Second, sample sizes necessary to detect interactions between unlinked markers (on different chromosomes) are far lower than those necessary for linked markers, where the production of different genotypes depends on recombination rates. Third, backcross progeny (unlike F_2 progeny) can be genotyped with dominant markers. In this study we use nonrecombinant backcrosses to address the following questions: how many chromosomes deviate from genotypic ratios expected under random assortment? Is there evidence of epistatic interactions among chromosomes and how extensive are these interactions? Are the observed genetic consequences of interpopulation hybridization congruent between different backcross hybrid lines differing in levels of evolutionary divergence? Do the genomic consequences of hybridization differ between sexes?

Materials and methods

Population sampling and culture maintenance

Populations were sampled from four southern California locations. Laguna Beach (LB, 33°33′N, 117°47′W) was sampled in October 1999 and again in November 2001. Royal Palms, Palos Verdes Peninsula (RP, 33°42′N, 118°19′W) was sampled in January 2000. San Diego (SD, 32°45′N, 117°15′W) was sampled in October 2001. Abalone Cove, Palos Verdes Peninsula (AB, 33°44′N, 118°22′W) was sampled in February 2002. All cultures were kept in a 20°C incubator with a 12 h light: 12-h dark cycle. Stock cultures were maintained in 400 mL beakers in natural seawater supplemented with commercial flake-type fish food and *Spirulina* algae.

Tigriopus californicus females mate only once and use stored sperm to fertilize multiple broods of offspring (Egloff, 1967; Vittor, 1971; Burton, 1985). Inbred lines

can therefore be created by isolating a single gravid female and allowing full siblings and their subsequent progeny to mate freely. Isofemale lines from the four populations were created by placing a single gravid female in a petri dish with filtered seawater (37 μm) containing 0.2 mg finely ground *Spirulina* per mL. Isofemale lines were maintained for a minimum of 2 months before experimental crosses were begun (minimum generation time is ~23 days at 20°C; Burton, 1987). Isofemale lines therefore underwent a minimum of two generations of full sibling mating with an expected heterozygosity reduction of at least 37.5% (Hedrick, 2000). Five isofemale lines were used for all subsequent crosses: AB40, RP14, LB15, LB75 and SD42.

Experimental crosses

In *T. californicus* mature males use their antennae to clasp virgin females and mate guard them until the females reach reproductive maturity (Egloff, 1967; Vittor, 1971; Burton, 1985). Virgin females can therefore be obtained by using a fine probe to tease clasped pairs apart under a dissecting microscope. In order to map molecular markers to individual chromosomes, a nonrecombinant backcross was established between isofemale lines RP14 and LB15 (hereafter referred to as the original mapping population). Virgin RP females were crossed with adult LB males, and the resultant F_1 females were backcrossed to LB males. Because recombination has not been observed in females (Ar-Rushdi, 1963; Burton *et al.*, 1981), crossing an F_1 female with an inbred parental male is a nonrecombinant backcross in which any nonparental two locus genotype indicates the markers are on different chromosomes.

In order to look at hybrid viability over different levels of population divergence, a larger set of nonrecombinant backcrosses was subsequently established between the same RP line (RP14) and isofemale lines SD42, LB75 and AB40 (hereafter referred to as the SD backcross, LB backcross and AB backcross). For these crosses, virgin females from the SD, LB or AB population were crossed with adult RP males, and the resultant F_1 females were backcrossed to males from the non-RP population. Mitochondrial DNA data (COI) show that the RP population is ~18% divergent from both the SD and LB populations, and ~1% divergent from the AB population. Populations LB and SD are ~10% divergent from each other (Edmands, 2001; D. Peterson, unpublished data; accession numbers AF096931, AF096932, AF096962, AF096963, AF096967 and AF096968).

All crosses were set up in sets of five females combined with five males. Algal rations were the same as for the isofemale lines. A total of ~40–50 pairs were set up for each of the four backcrosses. Dishes were checked three times per week. When females formed egg sacs they were transferred to a new Petri dish containing new seawater/*Spirulina*. Parental females were again transferred to a new dish when F_1 larvae hatched. After F_1 offspring formed clasped pairs, the pairs were dissected apart and F_1 females were transferred to a new dish containing males from the appropriate SD, LB, or AB isofemale line. Again, five females were united with five males in each dish. When F_1 females formed egg sacs they were transferred to a new dish. Adult females were again transferred to a new dish when backcross offspring hatched. Backcross offspring were frozen after reaching maturity. Offspring from different parents within the same backcross were pooled for subsequent molecular analyses.

Molecular methods

DNA was extracted from frozen individual copepods by standard proteinase-K digestion followed by phenol–chloroform–isoamyl alcohol extraction. Because of the small amount of starting tissue, each sample was split in two equal volumes prior to final DNA precipitation in an attempt to maximize the amount of DNA available for each of two future uses; one to be used for PCR of microsatellite loci, and the other for amplified fragment length polymorphisms (AFLPs).

Thirteen microsatellite loci were used in this study. Primers and PCR conditions for 11 loci are described in Harrison *et al.* (2004). One of these primer sets (TC1555) amplifies an additional locus (TC1555B) as a dominant marker in the RP and AB lines but not in the LB or SD lines. Finally, the 13th locus (TC62J8) is described in Edmands *et al.* (2005). For all PCR the forward primers were fluorescently labelled and run on a CEQ 8000 capillary sequencer (Beckman Coulter, Fullerton, CA, USA).

Three of the markers used were obtained by the AFLP method of Vos *et al.* (1995). MspI was used as the frequent-cutting and EcoRI as the rare-cutting restriction enzyme. Adapter and core primer sequences for the EcoRI end were those used by Vos *et al.* (1995), whereas those used for the MspI end were designed by Xu *et al.* (2000). Digestion of genomic DNA was performed by adding 40 μL of digestion mixture (5 units each of EcoRI and MspI in 10 mM Tris–HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, and 50 ng/μL BSA) to the dry DNA pellet and incubated at 37°C for 7 h. The digested fragments were then ligated to adapters by adding 10 μL of ligation mixture (5 pmol EcoRI adapter, 50 pmol Msp adapter, 1 mM ATP, 40 units T4 DNA ligase, 10 mM Tris–HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, and 50 ng μL BSA) and incubating at 16°C for 20–24 h.

Preselective amplification was carried out using 7 μL of ligation reaction as template DNA, EcoRI primer with no selective bases (E + 0) and Msp primer with one selective base (M + T).The PCR was performed in 25 μL final volume containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 25 pmol each primer and 0.75 units Taq polymerase. Cycling conditions consisted of 2 min at

72°C, followed by 25 cycles of 30 s at 94°C, 1 min. at 56°C and 1 min. at 72°C. Preamplification products were diluted 1 : 1 (v : v) with TE buffer, and then used as template DNA for selective amplifications.

Selective amplifications were conducted in volumes of 10 μL containing 5 μL of diluted preamplification product, 2.5 pmol EcoRI primers with two selective bases (E + AG), 10 pmol Msp primers with two additional selective bases (M + TAC), 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.35 units Taq polymerase. EcoRI primers were end-labelled with fluorescent dyes. Thermocycler conditions followed the touch-down procedure described by Vos *et al.* (1995). Selective amplification products were separated using the Beckman Coulter CEQ 8000 automated DNA sequencer.

Scoring and analysis

Genotypic data was obtained manually using the CEQ 8000 genotyping software. Molecular markers were first mapped to chromosomes by genotyping 11–22 individuals from the original RP–LB mapping population. Markers were then tested for amplification and/or presence (for AFLPs) in the SD and AB lines before use in experimental samples.

Deviations from 1 : 1 Mendelian ratios for each chromosome in each backcross were tested using contingency tables, the significance of which was assessed with a χ^2-test. Viabilities of homozygotes and their standard deviations relative to heterozygotes were calculated according to Haldane (1956) (also see Willett & Burton, 2003). With complete independence (no epistasis), two-locus genotypic frequencies should be predicted by multiplying the single-locus frequencies. Fit to this multiplicative model was assessed for each pair of loci using Fisher's exact test.

Results

Markers and mapping

Markers distinguishing chromosomal linkage groups in the three backcrosses are shown in Table 1. Chromosome numbers were assigned arbitrarily and do not indicate chromosome size, as this information is unknown. Our initial mapping efforts using 11–22 nonrecombinant LB backcross individuals resulted in 12 microsatellite loci mapped to nine chromosomes. An additional dominant marker amplified with the TC1555 primer set, and two AFLP markers (AGTAC258 and AGTAC323) mapped to three additional chromosomes for a total of 11 chromosomal linkage groups for the LB backcross. While analyzing the SD backcross an additional AFLP marker (AGTAC 214) was mapped to a twelfth chromosome, allowing all twelve chromosomes to be surveyed in this cross only. Only six of these markers were polymorphic between the RP and AB inbred lines, therefore only six chromosomes were surveyed in this cross. No markers were found to be specific to one sex.

Table 1 Molecular markers diagnostic for chromosomal linkage groups in each of the three backcrosses.

Chromosome	SD backcross	LB backcross	AB backcross
1	TCS558, TCS30	TCS558, TCS30	
2	TCS228	TCS228	
3	AGTAC258	AGTAC258	
4	AGTAC214		
5	TCS1203, TC62J8	TCS1203, TC62J8	TCS1203
6	TC1555B	TC1555B	
7	TC56J2	TC56J2	TC56J2
8	TCS480	TCS480, TCS61	TCS480
9	TC1814, TCS197	TC1814, TCS197	TCS197
10	TC1555	TC1555	TC1555
11	TC1202	TC1202	TC1202
12	AGTAC323	AGTAC323	

Microsatellite loci have the prefix 'TC' and amplified fragment length polymorphism loci have the prefix 'AGTAC'.

Genotypic data was obtained for 300 individuals in the SD backcross (169 males, 131 females), 301 individuals in the LB backcross (198 males, 103 females), and 301 individuals in the AB backcross (150 males, 151 females) for most markers. Notably, sex ratios were significantly male biased in the SD backcross ($\chi^2_1 = 4.8$, $P = 0.03$) and the LB backcross ($\chi^2_1 = 30.0$, $P = 0.0001$) but not the AB backcross ($\chi^2_1 = 0.003$, $P = 0.95$). Sample sizes in the SD backcross were 283–284 (165 males, 118 and 119 females) for the AFLP markers (chromosomes 3, 4 and 12) owing to PCR failure. Sample sizes in the LB backcross were 202 and 207 (142 and 146 males, 60 and 61 females) for the AFLP markers (chromosomes 3 and 12, respectively) owing to permanent loss of DNA sample during the ligation step of the AFLP process. It has previously been reported that there is no recombination in *T. californicus* females by Ar-Rushdi (1963) and Burton *et al.* (1981). For chromosomes in which two markers were scored we found low levels of recombinant genotypes in the samples scored for each cross. In the SD backcross 1.0% recombinants were observed for the chromosome 1 and 9 linkage groups, and 1.7% recombinants for the chromosome 5 linkage groups. In the LB backcross 0.33% recombinants were observed for the chromosome 9 linkage groups, 0.66% recombinants for the chromosome 1 and 5 linkage groups, and 1.33% recombinants for the chromosome 8 linkage groups. These data could possibly reflect either actual recombination events in females or mis-scoring. Nonetheless, these values are consistent with very restricted levels of recombination in females and we continue to use the term 'nonrecombinant' backcross throughout the paper. In addition, it should be noted that to simplify data presentation, genotypic data from only one of the markers from each linkage group is presented in this study. This is justified for two reasons: first the statistical

significance of the results did not differ depending on the marker used or if both markers from the questionable individuals were excluded from analysis, and second we used the marker that allowed us to be consistent with a other crosses where it was possible to use only one of the two markers e.g. chromosome 5 in Table 1.

Genetics of viability differences

A majority of the 12, 11 or six chromosomal markers deviated from expected Mendelian ratios in the three nonrecombinant backcross hybrid lines studied. Figure 1 presents the observed proportion of heterozygotes at each locus from adult individuals scored for each of the experimental backcrosses. In the SD backcross eight of 12 loci deviated significantly from the expected 1 : 1 genotypic ratio, while six of 11 loci were significant in the LB backcross, and four of six in the AB backcross. An excess of heterozygotes was responsible for the deviations in all cases except one. The most extreme case of heterozygote excess in both the divergent backcrosses was at chromosome 1 where 88% and 90% of the individuals scored were heterozygotes in the SD and LB backcrosses, respectively. General genotypic patterns were similar across chromosomes between the two divergent backcross lines. In the SD and LB backcrosses, eight of the 11 comparable markers showed similar patterns, meaning either that both conformed to expectations or both showed significant deviations in the same direction. Only three of six comparable markers exhibited similar genotypic patterns among all three backcross lines. Furthermore, regression analysis of single locus viabilities indicate significant concordance between the SD and LB backcrosses ($r^2 = 0.465$, $P = 0.02$), and lack of concordance between all other pairwise combinations (SD/AB $r^2 = 0.214$, $P = 0.36$; LB/AB is $r^2 = 0.205$, $P = 0.37$). However, when only the markers available for the AB comparison were used to assess concordance between SD and LB, the regression analysis is not significant ($r^2 = 0.434$, $P = 0.155$).

The proportion of heterozygotes in males vs. females for each backcross is presented in Fig. 2. In the SD backcross the sexes were significantly different at five chromosomal markers (2, 6, 10, 11 and 12). In the LB backcross chromosomal markers 5 and 11 differed significantly in heterozygosity levels between males and females. Chromosomes 3, 7, 9 and 10 also differed between sexes but were nonsignificant at $P = 0.05$. In all but one case where differences were observed within these two divergent crosses, females were the more heterozygous sex. Interestingly, the exception is chromosome 10 where males are significantly more heterozygous in the SD backcross and show a similar but nonsignificant trend in the LB backcross. This pattern of stronger heterozygote excess in females was largely absent in the AB backcross, with the only significant difference at chromosome 11 where males were more heterozygous than females. Averaged over all chromosomes females were more heterozygous than males in the LB and SD crosses, and the sexes were not significantly different in the AB backcross. Overall heterozygosity values in the LB backcross were 0.59 and 0.65 ($t_{1720} = -3.65$, $P = 0.0001$) in males and females, respectively. In the SD backcross these values were 0.61 and 0.67 ($t_{3357} = -3.58$, $P = 0.0002$). In the AB backcross these values were 0.55 and 0.52 ($t_{1803} = -1.24$, $P = 0.22$).

Genotypic count data were used to estimate single-locus homozygote viabilities relative to the heterozygote (Table 2). Relative viabilities ranged from 0.127 at

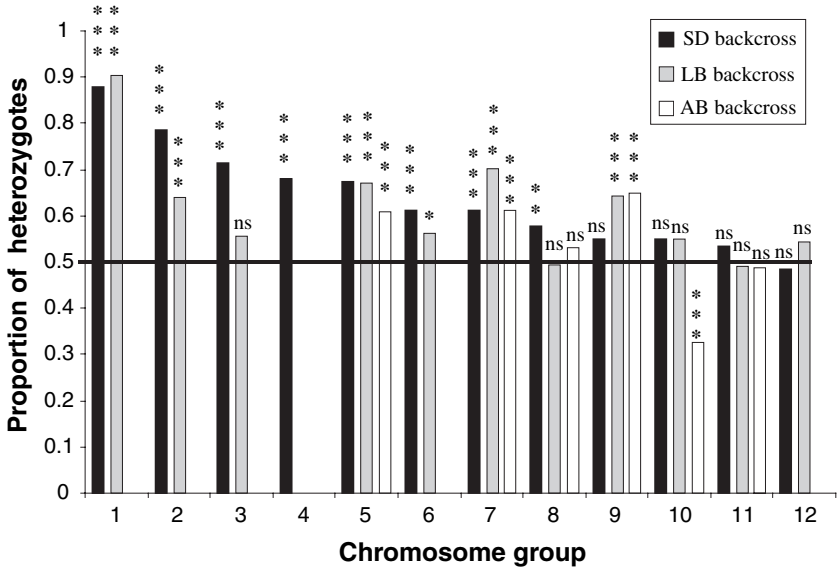

Fig. 1 Observed proportion of heterozygotes at 12 (SD backcross), 11 (LB backcross), and 6 (AB backcross) loci mapped to individual chromosomes. Asterisks indicate significance of chi-square tests for deviations from Mendelian genotypic ratios (*<0.05, **<0.01, ***<0.001). Solid black line indicates expected proportion of heterozygotes; 300 individuals were genotyped in the SD backcross except at chromosome 4 ($n = 284$), and chromosomes 3 and 12 ($n = 283$); 301 individuals were genotyped in the LB backcross except at chromosome 3 ($n = 202$) and 12 ($n = 207$); 301 individuals were genotyped in the AB backcross.

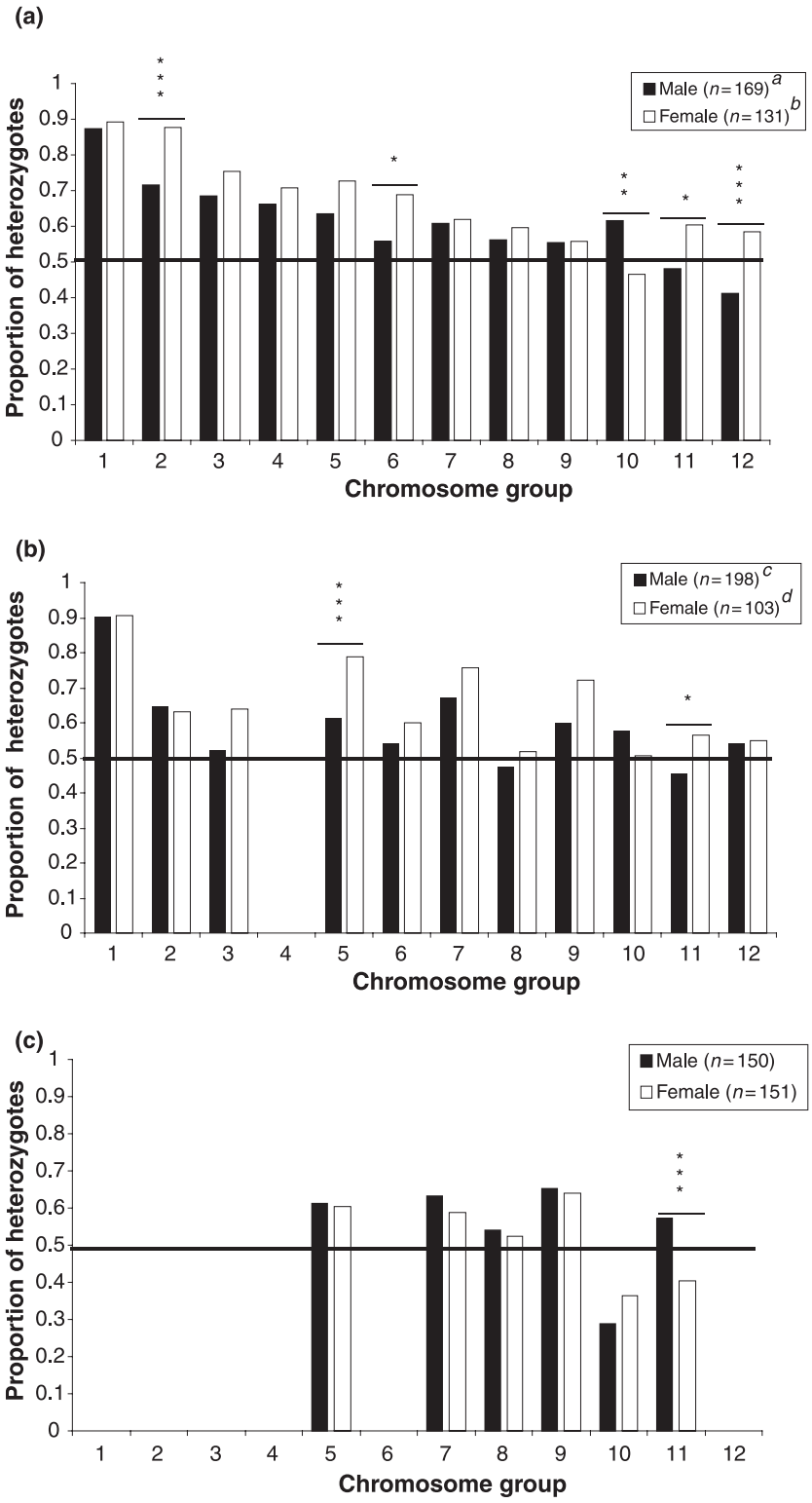

Fig. 2 Male vs. female proportion of heterozygotes in the SD backcross (a), LB backcross (b) and AB backcross (c). Asterisks indicate significance of *t*-test for differences in heterozygosity between sexes (*<0.05, **<0.01, ***<0.001). Solid black line indicates expected proportion of heterozygotes. [a]$n = 165$ for chromosomes 3, 4 and 12 for SD backcross males. [b]$n = 118$–119 for chromosomes 3, 4 and 12 for SD backcross females. [c]$n = 142$ for chromosome 3 and $n = 146$ for chromosome 12 for LB backcross males. [d]$n = 60$ for chromosome 3 and $n = 61$ for chromosome 12 for LB backcross females.

chromosome 1 to 1.05 at chromosome 12 in the SD backcross, from 0.106 at chromosome 1 to 1.03 at chromosome 11 in the LB backcross, and from 0.540 to 2.05 in the AB backcross. The average relative viability of the homozygote across all loci was 0.605 in the SD backcross, 0.670 in the LB backcross, and 0.963 in the AB

Table 2 Single locus relative viabilities (SD) based on genotypic ratios of backcross hybrid offspring.

Chromosome	1	2	3	4	5	6	7	8	9	10	11	12	Average	Range
SD backcross relative viability homozygote														
All	0.127	0.270	0.399	0.469	0.468	0.627	0.627	0.730	0.797	0.813	0.870	1.05	0.605	0.127–1.05
	(0.024)	(0.038)	(0.053)	(0.060)	(0.058)	(0.074)	(0.074)	(0.085)	(0.092)	(0.094)	(0.100)	(0.126)		
Male	0.133	0.393	0.456	0.509	0.560	0.789	0.635	0.771	0.789	0.619	1.07	1.41	0.678	0.133–1.41
	(0.032)	(0.067)	(0.077)	(0.084)	(0.089)	(0.122)	(0.100)	(0.119)	(0.122)	(0.098)	(0.165)	(0.222)		
Female	0.119	0.138	0.322	0.412	0.389	0.451	0.610	0.671	0.784	1.13	0.650	0.700	0.529	0.119–1.13
	(0.033)	(0.037)	(0.069)	(0.083)	(0.071)	(0.085)	(0.109)	(0.119)	(0.138)	(0.198)	(0.116)	(0.131)		
LB backcross relative viability homozygote														
All	0.106	0.557	0.793		0.488	0.776	0.425	1.01	0.549	0.808	1.03	0.829	0.670	0.106–1.03
	(0.021)	(0.067)	(0.110)		(0.060)	(0.090)	(0.054)	(0.117)	(0.066)	(0.094)	(0.118)	(0.117)		
Male	0.110	0.543	0.909		0.631	0.843	0.485	1.07	0.658	0.730	1.19	0.833	0.728	0.110–1.19
	(0.026)	(0.081)	(0.151)		(0.092)	(0.120)	(0.074)	(0.153)	(0.096)	(0.105)	(0.169)	(0.140)		
Female	0.095	0.576	0.550		0.268	0.651	0.316	0.891	0.368	0.962	0.763	0.794	0.567	0.095–0.962
	(0.033)	(0.118)	(0.147)		(0.065)	(0.131)	(0.073)	(0.176)	(0.082)	(0.190)	(0.152)	(0.206)		
AB backcross relative viability homozygote														
All					0.641		0.632	0.875	0.540	2.05	1.05		0.963	0.540–2.05
					(0.076)		(0.075)	(0.101)	(0.065)	(0.252)	(0.120)			
Male					0.623		0.573	0.841	0.525	2.43	0.736		0.955	0.525–2.43
					(0.105)		(0.097)	(0.138)	(0.090)	(0.437)	(0.122)			
Female					0.652		0.689	0.900	0.551	1.71	1.45		0.992	0.551–1.71
					(0.109)		(0.114)	(0.147)	(0.094)	(0.289)	(0.240)			

See Fig. 2 for explanation of sample sizes.

backcross. When taking into account only those chromosomes assessed in the AB backcross, the average relative viabilities were 0.718 for both the SD and LB backcrosses. In all, eight chromosomes (1–8), five chromosomes (1, 2, 5, 7 and 9), and four chromosomes (5, 7, 9 and 10) showed homozygous viabilities greater than two standard deviations from the heterozygote viability in the SD, LB and AB backcrosses, respectively.

Relative viabilities calculated separately for each sex are also presented in Table 2. Average viabilities of the homozygote across all loci were 0.15 and 0.16 higher in males than females in the SD and LB backcrosses. In the SD backcross, the viability of homozygotes was significantly lower (2 SD) relative to the heterozygotes at seven loci (1–5, 7 and 10) in males, and a remarkable 10 of 12 loci (1–8, 11 and 12) in females. Five (1, 2, 5, 7 and 9) and seven (1, 2, 3, 5, 6, 7 and 9) loci exhibited significantly lower homozygous viabilities relative to heterozygote viabilities in males and females of the LB backcross, respectively. In the AB backcross the average viability was only 0.037 higher in females than males. Four loci (5, 7, 9 and 10) and three loci (5, 7 and 9) were significantly different from the heterozygote in viability in males and females of the AB backcross, respectively.

Tests for locus × locus linkage disequilibrium were performed to assess levels of interaction among chromosomal linkage groups and the consequences of these interactions on genome integration in backcross hybrids. Relatively few cases of disequilibrium were detected in all three crosses (Table 3). Of a possible 132 pairwise comparisons in the SD backcross, six were significant and only two remained significant after correcting for multiple tests (chromosomes 3/12 and 4/9, $\chi^2_1 > 25$, $P < 0.00001$). In the LB backcross 110 pairwise

Table 3 Locus by locus tests of linkage disequilibrium. Shading indicates chromosome where markers were not available for the indicated backcross.

Interacting	SD backcross			LB backcross			AB backcross		
Chromosomes	All	Male	Female	All	Male	Female	All	Male	Female
2/5	*	*							
2/11	*	*							
3/1	**	*	*						
3/4	*								
3/12	***	***	*	***	***	***			
4/9	***	***	***						
4/11		*							
5/8		*							
12/9		**							
5/3				*					
5/9				*					
5/10				***					
12/1					**				
12/7				*	*				
12/10					*				
12/11						*			
1/11				**					
7/10					*				
8/9						**	***	***	***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

comparisons were possible and six were significant. Only one remained significant after correcting for multiple tests (chromosomes 3/12, $\chi^2_1 > 25$, $P < 0.00001$). The three significant cases were due to a lower than expected frequency of homozygous/heterozygous genotypes. In the AB backcross 30 pairwise comparisons were possible of which one was significant, although this interaction was not significant after Bonferroni correction. All detected cases of disequilibrium were unique to each backcross with the exception of the chromosome 3 by chromosome 12 comparison between the SD and LB backcrosses. Results of linkage disequilibrium tests also differed slightly between sexes within the SD and LB backcrosses, but not within the AB backcross. In the SD backcross eight significant interactions were detected in males and only three in females. Only two interactions in males (chromosomes 3/12 and 4/9) and one in females (4/9) were significant after correcting for multiple tests. Similarly five significant interactions were detected in LB backcross males and three in females. One of these interactions (chromosomes 3/12) was significant after correcting for multiple tests in both males and females.

To extend our analysis beyond interactions between pairs of loci, we calculated the probability of all 12, 11 or six locus genotypes (for SD backcross, LB backcross and AB backcross, respectively) based on observed heterozygosity. The probabilities of all genotypes within a class (i.e. 0 heterozygous loci, one heterozygous locus, two heterozygous loci, etc.) were then summed to obtain the probability of each class. From this we can calculate the expected number of individuals with 0 heterozygous chromosomes, one heterozygous chromosomes, two heterozygous chromosomes, etc. under the assumption that all chromosomes are independent (no interactions). The observed distribution of the number heterozygous chromosomes per individual differed significantly from the expected frequencies in the SD backcross ($\chi^2_{12} = 23.1$, $P = 0.027$), but not the LB backcross or AB backcross ($\chi^2_{11} = 12.8$, $P = 0.306$). Frequency distributions for the SD and LB backcrosses are shown in Fig. 3.

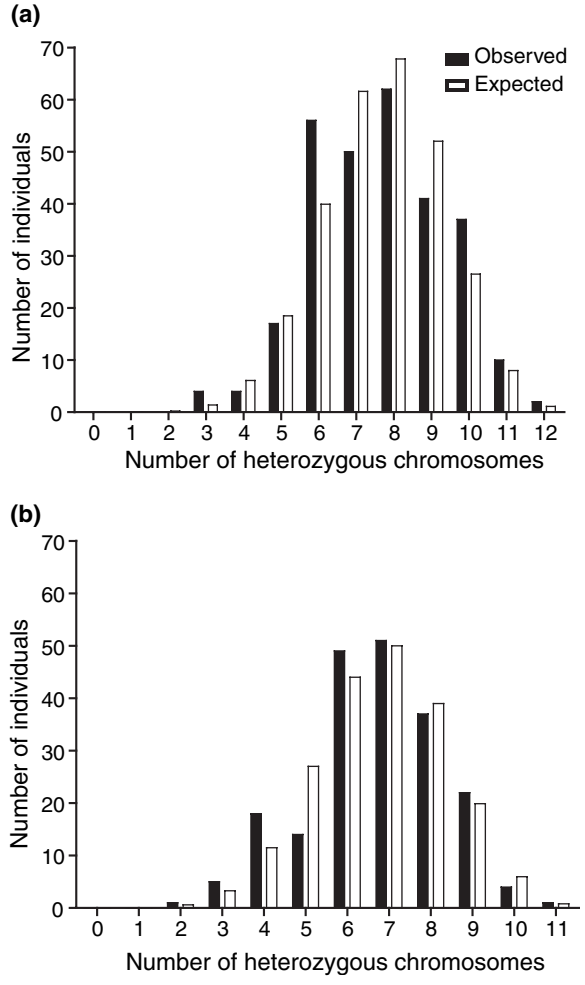

Fig. 3 Observed and expected numbers of individuals heterozygous for 0–12 chromosomes in the SD backcross (a), and 0–11 chromosomes in the LB backcross (b). Expected values calculated assuming independence of loci. The frequency distribution of the observed SDBC data differed from the expected values ($\chi^2_{12} = 23.1$, $P = 0.027$), the distribution of the observed LBBC data was not significantly different from expected values ($\chi^2_{11} = 12.8$, $P = 0.306$).

Discussion

This experiment stems from previous investigations demonstrating that: (i) crosses between populations of *T. californicus* typically result in F_1 heterosis and F_2 (or backcross) hybrid breakdown for a variety of fitness related measures including survivorship (Burton, 1987, 1990; Burton *et al.*, 1999; Edmands, 1999); (ii) the magnitude of hybrid breakdown is strongly correlated with evolutionary divergence (Edmands, 1999); (iii) negative epistatic interactions play a significant role in hybrid breakdown (Edmands, 1999); and (iv) quantitative genetic and two-locus studies have shown that negatively interacting loci can reside on different chromosomes (Burton, 1987; Edmands, 1999). These previous investigations have provided significant information pertaining to the mechanisms and manifestations of hybrid breakdown. However, numerous questions remain concerning the genetics involved. For example, we know that epistatic interactions are important, and that interacting loci can reside on different chromosomes, but questions addressing the number and distribution of loci involved remain unanswered. In this study we have taken the approach of using mapped molecular markers to determine the viability effects of intact chromosomes. This study provides a first assessment of the sum of genome-wide effects on viability and genetic architecture in *T. californicus* interpopulation hybrids.

If all loci assort randomly, segregation of genetic markers are expected to result in 1 : 1 genotypic ratios

in nonrecombinant backcross hybrids. Patterns of deviation from this ratio give us clues into the genetic mechanisms involved in reduced hybrid fitness. Skewed genotypic ratios in the direction of heterozygote excess can be the result of a number of hybrid phenomenon. Segregation distortion during gamete formation could contribute to this pattern, but does not explain the observed differences between males and females. Data for the reciprocal backcross (not collected in this study) would be needed to definitively test for segregation distortion. Deviant ratios may also be caused by a disparity in success of gametes during fertilization. Here, alleles favoured in a hybrid genetic background may represent selfish genes that enhance success of gametes even though they carry a significant fitness cost in adults (Rieseberg *et al.*, 2000). Finally, differential zygote viability may account for the significant deviations from Mendelian expectations. Although our experimental design does not allow us to distinguish between gamete and viability selection, several studies have demonstrated that zygotic mortality is significant in hybrid offspring of divergent interpopulation crosses (Burton, 1986, 1987; Edmands, 1999). Further, Willett & Burton (2001) showed that genotypic ratios for the cytochrome *c* gene were skewed in adult *T. californicus* hybrids but not in newly hatched larvae, indicating that the observed deviations from Mendelian ratios were caused by differential viability rather than gametic selection. Results from these previous studies suggest that differential zygote viability is likely an important cause of deviant Mendelian ratios observed here.

There are several potential sources of differential zygote viability in the *T. californicus* system. First, RP alleles might be favoured under laboratory conditions. However, Edmands *et al.* (2005) found that hatching and survival numbers were significantly lower for RP than SD populations raised for two generations under similar laboratory conditions as those in this study, suggesting that this is not a likely explanation, at least for the SD backcross. A second possibility is the alleviation of high genetic load (Myburg *et al.*, 2004). The natural history of *T. californicus* may drive populations to carry a significant genetic load. Populations inhabit a highly variable environment and drastic fluctuations in population size have been observed. This suggests that populations experience frequent and repeated bottlenecks, but the magnitude and genetic consequences of these bottlenecks is unknown. Burton & Lee (1994) reported low within population heterozygosity values at five allozyme loci across 10 *T. californicus* populations ($H = 0.07 \pm 0.02$). Edmands & Harrison (2003) also reported that overall genetic variation within natural *T. californicus* populations is low, with expected heterozygosity for five microsatellite loci ranging from 0.138 to 0.331 (although it should be noted that both of these estimates are upwardly biased as only polymorphic loci were used in analyses). This data suggests that drift is likely a strong force driving the accumulation of deleterious recessives within populations, while inbreeding may not be extensive enough to purge them. Experimental evidence that *T. californicus* populations carry a considerable genetic load comes from studies showing interpopulation F_1 hybrids exhibit slight heterosis (Edmands, 1999) and full-sib matings result in varying degrees of inbreeding depression (Palmer & Edmands, 2000).

Our data suggests that the masking of deleterious recessives plays a significant role in the resulting genetic architecture of backcross hybrids. Consider the situation where any given marker used in this study is tightly linked to a locus with a deleterious recessive allele segregating in the SD, LB and/or AB isofemale lines. Given that both parents of the backcross hybrid (F_1 female and SD, LB or AB male) can be heterozygous for the deleterious recessive, the resulting frequencies of viable genotypes at the mapped marker are 67% heterozygotes and 33% homozygotes. This pattern is consistent with average levels of heterozygosity observed in the SD backcross (0.638, relative viability 0.605) and the LB backcross (0.614, relative viability 0.670). However, there is some evidence that all of the observed heterozygote excess is not attributable to the masking of deleterious recessives.

Deleterious epistatic interactions also likely play a role in differential zygote viability. It is widely accepted that intrinsic post-zygotic barriers to gene flow are in large part due to negative epistatic interactions between hybrid genomes (i.e. Dobzhansky–Muller interactions) (Coyne & Orr, 1997; Turelli *et al.*, 2001; Presgraves, 2003). We found a number of significant pairwise interactions between chromosomes that implicate negative epistasis between heterozygote and homozygote genotypes. Our results are consistent with low numbers of pairwise interactions detected in other marker-based studies of hybrid incompatibilities (Myburg *et al.*, 2004; Slotman *et al.*, 2004). Furthermore, the hypothesis that all chromosomes are acting independently was rejected in the SD backcross suggesting some multilocus epistatic interactions. These findings are congruent with theory, and an increasing number of observations that complex epistatic networks are much more common than simple interactions between pairs of genes (Orr, 1995; Rieseberg *et al.*, 1996; Jiang *et al.*, 2000; Myburg *et al.*, 2004). The exception to this observation being a number of observations of single autosomal loci interacting with the X-chromosome (e.g. Slotman *et al.*, 2004), a scenario not possible in *Tigriopus* where there is no heterogametic sex (Ar-Rushdi, 1963; Lazzaretto & Libertini, 1986).

The drastic reduction of parental homozygotes at chromosome 1 implies a situation similar to that of synthetic lethals or synthetic deleterious loci (Phillips & Johnson, 1998). One interpretation of this data is that loci on this chromosome are involved in strong negative epistatic interactions with other areas of the hybrid genome. An equally likely interpretation is that there are

a large number of deleterious alleles segregating on this chromosome in both the SD and the LB populations. Our experimental design does not allow us to distinguish between the two scenarios (although they are not mutually exclusive). The similar and dramatic results at chromosome 1 in the two divergent crosses make it an enticing area of the genome in which to look for loci of large viability effects in future studies of these hybrids.

Characteristics of our observed data combined with our experimental design may prevent us from detecting a larger number of interactions among chromosomes. First, the backcross design used here allows us only to test for homozygote × heterozygote interactions. However, Edmands (1999) using a quantitative genetic approach found that nonrecombinant backcrosses exhibited reduced fitness due to a significant epistatic component suggesting that homozygote × heterozygote interactions are prevalent. Second, the extensive heterozygote excess observed in our data suggests a significant role for dominance, and if both masking deleterious recessives and negative epistatic interactions are driving distorted genotypic ratios we may not be able to partition the two in the current investigation. Comparing recombinant and nonrecombinant crosses in future studies may give greater insight as to the presence and location of interacting loci.

A number of studies are finding evidence of correlated patterns of introgression among both laboratory and natural hybrids, suggesting that selection rather than chance is governing the genomic consequences of hybridization (Rieseberg *et al.*, 1996, Wilding *et al.*, 2001). Our results are at least partly consistent with this finding. At least for the two divergent crosses, effects of individual chromosomes (an exceptional case is chromosome 1) are remarkably similar. This congruence cannot be attributed to (i) simple differences in chromosome size because the 12 chromosomes are nearly isomorphic (Ar-Rushdi, 1963), (ii) shared nuclear-cytoplasmic interactions (because the three backcrosses have different cytoplasmic backgrounds) or (iii) extensive shared evolutionary history (populations LB and SD are ~10% divergent in mitochondrial DNA (Edmands, 2001; D. Peterson, unpublished data).

The locus-by-locus comparisons showing evidence of disequilibrium were fairly incongruent among the three hybrid lines. However, it is interesting to note that one of the two interactions significant after corrections for multiple tests was shared between the LB and SD backcrosses (3/12), but was unable to be tested in the AB backcross. The only significant interaction in the AB backcross was unique to this cross.

Sex also appears to play a significant role in hybrid viability. We found that overall, in the two divergent crosses (SD and LB backcrosses) females were more heterozygous and that the homozygous genotype exhibited relative viabilities 15–16% lower than those in male hybrids. Similarly, sex ratios were significantly male-biased in the SD backcross (56% male) and the LB backcross (66% male) but not the AB backcross (50% male). While male bias is common in *Tigriopus*, bias particularly in the LB backcross exceeds previous reports for primary sex ratio (49–58% male, Voorduow & Anholt, 2002a), further suggesting higher mortality in homozygous hybrid females.

In looking at relative viabilities of the cytochrome *c* locus in *T. californicus*, Willett & Burton (2001) found significant heterogeneity between sexes in two different interpopulation F_2 hybrid crosses, and a third cross was nearly significant. Unlike our results, none of these differences was attributable to heterozygote excess. Sex-specific consequences of hybridization are difficult to interpret given our lack of knowledge regarding the sex determining mechanism(s) in *T. californicus*. *Tigriopus californicus* lacks a morphologically distinguishable sex chromosome (Ar-Rushdi, 1963), and Voorduow & Anholt (2002a,b) have suggested that sex determination is polygenic with a strong environmental (temperature) influence.

One explanation for reduced viability of homozygous females is that females may be hemizygous for one or more sex-determining factors, and these factors, or loci closely linked to them, may have negative epistatic interactions with homozygous loci. While we do not know for sure which sex is heterogametic, females are the more likely candidates given the correlation between restricted recombination and heterogamety in other taxa (Bull, 1983). An alternative explanation for reduced homozygote viability in females is gender-specific susceptibility to inbreeding. There are certainly examples of sex differences in genetic load affecting fertility (e.g. Saccheri *et al.*, 2005), but there is less evidence for differences affecting viability (e.g. Eanes *et al.*, 1985). A more thorough understanding of the distribution and mechanics of the 'sex determining factors' in *T. californicus* will contribute significantly to the interpretation of our results.

In summary, we found that interpopulation crosses resulted in significant deviations from expected Mendelian ratios at a high proportion of the loci studied suggesting that most chromosomes carry incompatibilities. Although one might expect significant barriers to genome integration between populations with approximately 18% mitochondrial sequence divergence, lower relative viabilities of the parental homozygous genotype was the rule rather than the exception. One implication of this pattern is that populations of *T. californicus* carry a significant genetic load, and masking deleterious recessives significantly affects hybrid viability. The two divergent crosses showed moderately congruent viability effects across all comparable loci, and neither showed significant congruence with the less divergent AB backcross. Epistatic effects between pairs of chromosomes appeared to be weak compared to the effects of individual chromosomes. However, future comparisons of

recombinant and nonrecombinant backcrosses may allow a better partitioning of heterozygote excess (dominance) and epistatic interactions. Finally, genomic consequences of interpopulation hybridization differ between sexes indicating either hemizygote-homozygote interactions or sex specific genetic load affecting viability.

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