JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12913

Recovery from hybrid breakdown in a marine invertebrate is faster, stronger and more repeatable under environmental stress

A. S. HWANG, V. L. PRITCHARD[1] & S. EDMANDS

Department of Biological Sciences, University of Southern California, Los Angeles, CA, USA

Keywords:

copepod;
experimental evolution;
hybrid viability;
Tigriopus californicus.

Abstract

Understanding how environmental stress alters the consequences of hybridization is important, because the rate of hybridization and the likelihood of hybrid speciation both appear elevated in harsh, disturbed or marginal habitats. We assessed fitness, morphometrics and molecular genetic composition over 14 generations of hybridization between two highly divergent populations of the marine copepod *Tigriopus californicus*. Replicated, experimental hybrid populations in both control and high-salinity conditions showed a decline in fitness, followed by a recovery. Recovery was faster in the salinity stress treatment, returning to parental levels up to two generations earlier than in the control. This recovery was stable in the high-salinity treatment, whereas in the control treatment, fitness dropped back below parental levels at the final time point. Recovery in the high-salinity treatment was also stronger in terms of competitive fitness and heat-shock tolerance. Finally, consequences of hybridization were more repeatable under salinity stress, where among-replicate variance for survivorship and molecular genetic composition was lower than in the control treatment. In a system with low effective population sizes (estimates ranged from 17 to 63), where genetic drift might be expected to be the predominate force, strong selection under harsh environmental conditions apparently promoted faster, stronger and more repeatable recovery from depressed hybrid fitness.

Introduction

Hybridization between populations and species is an increasingly important issue for conservation, due both to intentional translocations aimed at 'genetic rescue' (Miller *et al.*, 2012; Heber *et al.*, 2013) and to accidental mixing caused by introduced species, introgression between farmed and wild populations and other anthropogenic mishaps (Ellstrand & Schierenbeck, 2000; Allendorf *et al.*, 2001; Hasselman *et al.*, 2014). Hybridization is also increasingly recognized as a creative evolutionary force potentially leading to hybrid speciation (Rieseberg *et al.*, 1999; Mallet, 2007; Dittrich-Reed & Fitzpatrick, 2013; Keller *et al.*, 2013; Trier *et al.*, 2014). Of particular interest is how the combination of hybridization and environmental stress may alter fitness consequences of gene interactions (both within and between loci) and rates of adaptation.

Some studies have found that environmental stress aggravates hybrid incompatibilities (Ellison & Burton, 2008; Koevoets *et al.*, 2012; Barreto & Burton, 2013). However, in other studies, extrinsic stress has been shown to decrease the detrimental effects of hybridization (Armbruster *et al.*, 1997; Edmands, 2007; Willett, 2012), possibly because stress can cause the benefits of within-locus hybridity to outweigh the detriments of between-locus hybridity (Armbruster *et al.*, 1997). Hybridization may also accelerate adaptation to stressful or novel habitats as advanced generation hybrids are expected to have high variance in fitness even if mean fitness is depressed. In some cases, the 'best' recombinant hybrids may have fitness exceeding both parents due to positive epistasis and/or additive effects of combining beneficial alleles (Rieseberg *et al.*, 1999; Dittrich-

Correspondence: Suzanne Edmands, Department of Biological Sciences, University of Southern California, 3616 Trousdale Parkway, Los Angeles, CA 90089, USA.
Tel.: +1 213 740 5548; fax: +1 213 740 8123; e-mail: sedmands@usc.edu
[1]Present address: Department of Biology, University of Turku, Turku 20014, Finland.

© 2016 EUROPEAN SOCIETY FOR EVOLUTIONARY BIOLOGY. *J. EVOL. BIOL.* **29** (2016) 1793–1803
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Reed & Fitzpatrick, 2013; Edmands, 2015). Interactions between hybridization and environmental stress are particularly important for conservation, as hybridization often occurs in marginal or degraded habitats (Allendorf *et al.*, 2001) where early generation hybrids may be favoured (Maschinski *et al.*, 2013; Ryan *et al.*, 2013). Over longer timescales, there appears to be an association between hybrid speciation and stressful or novel habitats (Nolte *et al.*, 2005; Mallet, 2007; Arias *et al.*, 2008; Donovan *et al.*, 2010).

While some hybrid lineages are successful, particularly in challenging habitats, fairly little is known about the repeatability of hybridization effects on population fitness or molecular composition (Edmands, 2015). This lack of knowledge is due, in part, to the scarcity of studies assessing outcomes of replicated contact between different lineages (Abbott *et al.*, 2013; Hoskin & Higgie, 2013). Outcomes might be expected to be altered by temporal or spatial environmental contingencies and by genetic drift, particularly if early-generation hybrid fitness is low or has a complex genetic basis, increasing fitness variance among recombinant genotypes. Despite these contingencies, a few studies have reported repeatable long-term consequences of hybridization (Rieseberg *et al.*, 1996; Brochmann *et al.*, 2000; Wang *et al.*, 2001; Schwarzbach & Rieseberg, 2002), attesting to the strength of selection on hybrid gene combinations.

Our study looked at long-term consequences of hybridization in the marine copepod *Tigriopus californicus* under control vs. salinity stress conditions. Replicated, experimental hybrid swarms were established using two genetically differentiated populations (18% divergence for mitochondrial COI, Peterson *et al.*, 2013; 4% divergence for nuclear-encoded mitochondrial Rieske iron–sulphur protein, Willett & Ladner, 2009). While these two populations are more divergent than many recognized species, they fall within the same mtDNA clade (one of six mtDNA clades in the species; Peterson *et al.*, 2013) and are reproductively compatible, producing viable and fertile progeny over multiple generations (Edmands *et al.*, 2005; Hwang *et al.*, 2011). Crossing these two populations is known to cause significant F2 hybrid breakdown for viability (Hwang *et al.*, 2011), a phenomenon known to have a complex genetic basis in this species (Pritchard *et al.*, 2011; Foley *et al.*, 2013). This study was maintained for 14 discrete generations, using up to 100 gravid females to initiate each replicate in each generation. Replicates were periodically assessed for fitness, morphology and molecular composition. In this way, we were able to follow the temporal pattern and repeatability of hybridization effects, with and without salinity stress, in a controlled laboratory environment that minimized environmental contingencies. This allowed us to test several predictions about the interaction between hybridization and extrinsic stress. Because stress can shift the relative impacts of beneficial within-locus effects vs. detrimental between-locus effects, we predicted reduced hybrid fitness problems under salinity stress. Further, because environmental stress imposes stronger selection, we predicted recovery from hybrid fitness problems to be both faster and more repeatable under salinity stress. To our knowledge, this is the first experimental, multigeneration study of the strength and repeatability of changes in hybrid fitness in control vs. stressed conditions.

Materials and methods

Reproductive biology of *Tigriopus californicus*

The species is an obligately sexual, harpacticoid copepod whose reproductive biology has been well studied (Burton, 1985). Males clasp virgin females and mate guard them until the female becomes reproductively mature. The male then inseminates the female before releasing her and the female uses stored sperm to fertilize multiple clutches of eggs. Eggs hatch into larvae, which metamorphose into juveniles, which then develop into morphologically distinguishable adults.

Population sampling

Populations were sampled from two southern California locations, Royal Palms (RP, 33°42′N, 118°19′W) and San Diego (SD, 32°45′N, 117°15′W), in December 2005. These two populations show approximately 18% mitochondrial cytochrome oxidase I divergence (Peterson *et al.*, 2013). Samples were maintained as mass cultures in 400-mL beakers with 350 mL filtered sea water (37 μm) and algal food supplements and housed in a 20 °C incubator with a 12-h light: 12-h dark cycle.

Pilot studies

Prior to the set-up of the experiment, a 14-days test showed that the growth rates of algae (*Platymonas* sp.) in the two salinity conditions were not significantly different. A pilot study also showed that *T. californicus* growth and/or development (measured as size at 2 weeks) were compromised at 53 ppt (Fig. S1).

Establishment and maintenance of hybrid swarms

In January 2006, three different population treatments (100% RP, 100% SD and 50% RP:50% SD) were initiated in each of two salinities (35 ppt control conditions, C, and 53 ppt salinity stress conditions, S). Twelve replicates of each population/salinity treatment were established by placing 100 gravid females in each 400-mL beaker containing 300 mL filtered sea water, 50 mL live *Platymonas* culture and finely ground Spirulina and Tetramin flakes at a concentration of 0.2 mg mL^{-1}.

Beakers were arranged in a randomized fashion in one 20 °C incubator set to a 12-h light: 12-h dark cycle. Beakers were monitored each week for the presence of juvenile copepods. If juveniles were present, all adult females were removed from the beaker. One week later, all adult females were again removed. This was performed to confirm that all females of the previous generation were removed. Beakers were then monitored once a week, and, once adult females with eggs were observed, they were transferred to new beakers with fresh culture medium to start the next generation. Adult gravid females were transferred to the new-generation beaker for the next 2–3 weeks depending on the presence or absence of new juveniles. When juveniles were observed in the new beaker, adult females were removed. In this way, generations were maintained as discrete. Once a week, all beakers were fed and rotated within the incubator to homogenize light exposure. For selected generations, up to 20 gravid females and 20 mature males were removed from each beaker and used for morphometric assays and fitness assays (females only). All copepods were either returned to their source beakers after assays were completed, or frozen for molecular analysis. Replicates were maintained for up to 14 generations (18 months).

Development time and survivorship assays

For each generation scored (generations 1, 2, 3, 5, 7, 9, 11 and 13), 20 gravid females were sampled from each replicate and isolated into individual Petri dishes containing 11 mL filtered sea water supplemented with ground Spirulina and Tetramin flakes. Females with red egg sacs (red eggs being more mature and therefore closer to hatching) were preferred to those whose eggs were still green in colour. Each dish was monitored three times per week until eggs hatched. After hatching, 10 larvae per clutch were pipetted into a new Petri dish with fresh culture medium. Development time is expected to increase under harsh conditions (Fig. S1). To assess development time in the long-term cultures, we recorded two different measures 7 days after hatching: number of individuals metamorphosed into adults and the presence or absence of larvae (the presence of larvae indicating slow development). Fourteen days after hatching, all individuals in each dish were counted to determine survivorship (= number of live individuals at day 14/number of larvae in dish at day 0).

Morphological assays

Morphometric assays were also performed at generations 1, 2, 3, 5, 7, 9, 11 and 13. Measurements were taken from digital images of adult copepods following procedures in Edmands & Harrison (2003). As each sampled generation matured, up to 20 females and 20 males were randomly chosen from each replicate. All measurements were performed at a magnification of 32× using a Leica MZ12 dissecting scope (Buffalo Grove, IL, USA). Digital images were captured and morphological measurements were taken using Optimas 5.2 (Meyer Instruments, Inc., Houston, TX, USA). Absolute size was calibrated using a stage micrometre. Eight measurements were taken for males (cephalothorax length, cephalothorax width, urosome length, urosome width, telson width, caudal seta length, antennule width and clasper width) and six were taken for females (cephalothorax length, cephalothorax width, urosome width, antennule width, egg sac length and egg sac area).

Competitive fitness assays

After 14 generations of hybridization, the competitive ability of surviving replicates was determined to gain a more comprehensive measure of overall fitness. Because individuals from different populations are indistinguishable by eye, and hybrid populations will share alleles with parental populations, it was not possible to compete different treatments directly against each other. Instead, a third, genetically divergent population from Santa Cruz, CA (36°57′N, 122°03′W) was used for this competition assay. Generation 14 females from selected replicates were isolated until their clutch hatched. In a Petri dish containing 55 mL sea water of the experimental replicate salinity, one complete Santa Cruz (SC) clutch and one complete experimental clutch were combined. Thirty plates were set up from each treatment for a total of 180 plates. Plates were incubated in a 20 °C incubator with a 12-h light: 12-h dark cycle and fed once a week by adding five drops of food mixture (0.2 g finely ground Spirulina and Tetramin in 100 mL filtered sea water) with a Pasteur pipet. After 3 weeks, 10 surviving individuals from each plate were frozen for microsatellite identification. Five diagnostic microsatellites (loci 30, 558, 228, 1203 and 1555; see Genotyping section below) were utilized to identify the population of origin for each sampled individual.

Heat-shock assays

Heat -shock assays were performed on offspring of generation 13 females. Prior to the assays, a pilot stress test determined that 20 min at 36 °C reduced survivorship but was not 100% lethal. For heat-shock assays, one male and two virgin females from a generation 13 treatment were placed together in a Petri dish. One hundred and fifty of these families were set up for each treatment. Dishes were monitored until males fertilized both females. When egg sacs were observed, females were isolated to their own dish. When eggs hatched, 10 larvae from each clutch were isolated to a new dish. Seven days later, dishes were exposed to a heat shock

of 36 °C for 20 min. Dishes were returned to incubation temperatures for 1 h, and survivors were counted.

Genotyping

At generations 7 and 13, a maximum of 40 individuals (20 females, 20 males) from each hybrid replicate were genotyped for one mitochondrial locus (cytochrome oxidase 1, COI) and 11 nuclear microsatellite loci (chromosome number_locus name: 1_30, 1_558, 2_228, 5_1203, 5_62J8, 7_56J2, 8_480, 9_197, 9_1814, 10_1555, 11_1202). The average number of individuals genotyped was 38.3 per replicate, with a range of 28–40. At generation 13, the same loci were genotyped in up to 10 individuals from each control replicate to test for any contamination among beakers. The mitochondrial (COI) locus was amplified using primers COIVHel (5′-TACACCTCAGGATGTCCAAAAAATCA-3′) and COIVLel (5′-GAGGGGCTACGAACCACAAAGATA-3′) modified from (Folmer *et al.*, 1994). Polymerase chain reactions were carried out in 12 μL volumes containing 0.5 μL template DNA, 1 μM forward primer, 1 μM reverse primer and 2.5 mM $MgCl_2$. Temperature cycling was as follows: 5 min denaturation at 94 °C, 40 cycles of 30 s at 94 °C, 35 s at 50 °C and 1 min at 72 °C, followed by 5 min at 72 °C. PCR products were restriction enzyme digested by HinfI at 60 °C for 1 h. HinfI cleaves a restriction site present in the RP COI sequence, but not the SD sequence. Restriction digests were run on 1.2% agarose gels for 1 h at 100 V. For microsatellite assays, protocols followed Hwang *et al.* (2011) and mapping information is included in Harrison & Edmands (2006) and Pritchard *et al.* (2011). For a genomewide analysis, up to 40 individuals (20 females, 20 males) from each hybrid replicate in generation 11 were also screened for 31 SNP loci (Appendix S1) spanning all 12 chromosomes and the mitochondria. SNP development, assay protocols and mapping information are described in Pritchard *et al.* (2011) and Foley *et al.* (2011).

Data analyses

Analyses of fitness and morphological characters within and between experimental population treatments were done using Statistica 7.1 (StatSoft, Tulsa, OK, USA). Nested analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) were used to quantify differences in measures among the different experimental population treatments and replicates. When appropriate, Bonferroni *post hoc* tests were utilized to determine the statistical significance between group means. To compare each experimental replicate as well as all replicates of a population type to the midparent value (the average of the two parental means), we conducted ANOVAS followed by planned contrast tests. We focused on comparisons to the midparent (e.g. Lynch, 1991) because we were specifically interested in the nonadditive effects (dominance and epistasis) induced by hybridization. The overall effect of salinity treatment on hybrid fitness trajectories was assessed by repeated--measures ANOVA, with treatment (control vs. high salinity) as the categorical predictor variable, and both generation (1, 2, 3, 5, 7, 9 and 13) and fitness metric (survivorship proportion and metamorphosis proportion) as repeated-measures factors. Morphological hybrid index scores were calculated following Carney *et al.* (2000), such that a score of 0 indicates RP-like morphology whereas a score of 1 indicates SD-like morphology.

For molecular data, GENEPOP 4.2 (Raymond & Rousset, 1995; Rousset, 2008) was used to calculate allele and genotype frequencies, deviations from Hardy–Weinberg equilibrium (both single-locus and global *U*-tests), pairwise F_{ST} between replicates and genotypic disequilibrium between pairs of loci. To further test for nuclear–nuclear epistatic interactions, two-locus nuclear genotypes were pooled into four categories across all physically unlinked loci: matched homozygotes, homozygote–heterozygotes, heterozygote–heterozygotes and unmatched homozygotes. Observed two-locus genotype numbers were compared to expected numbers determined by multiplying single-locus ratios. Paired two-tailed *t*-tests were used to compare observed vs. expected genotype numbers. To test for mitonuclear epistasis, observed numbers of matched (nuclear homozygotes with mitochondrial haplotype from the same population) and unmatched (nuclear homozygotes with mitochondrial haplotype from the alternative population) genotypes were compared to expected two-locus genotype numbers (determined by multiplying single-locus ratios), with significance tested by one-tailed *t*-tests. Effective population size was estimated for each replicate for which microsatellite data were collected at both generations 7 and 13. Estimates were obtained with Nb_HetEx (Zhdanova & Pudovkin, 2008) using the temporal method of (Waples, 1989). Population differentiation in each generation was assessed with locus-by-locus analysis of molecular variance (AMOVA) run with Arlequin 3.5 (Excoffier *et al.*, 2005) using 10 000 permutations. Hybrid index scores for each individual were calculated by assigning 0 for each RP allele and 1 for each SD allele. Alleles shared between populations (found in microsatellite loci 558, 197 and 56J2) were given a score equal to the probability that the allele came from SD. Individual hybrid index was determined by averaging over the number of loci scored for each individual.

Results

Phenotypic data

The high-salinity (S) treatment of 53 ppt was more stressful than the control (C) treatment of 35 ppt. The

pilot study (Fig. S1) showed lower growth rate at 53 ppt than at 35 ppt. In the swarms experiment itself, development was also slower in the high-salinity treatment. Here, surveys performed 7 days after hatching showed a higher proportion of clutches still containing larvae in the high-salinity treatment throughout the 13 generation experiment, meaning that not all individuals had metamorphosed to juveniles (Fig. S2).

In the swarms experiment, the majority of replicates, including both pure and hybrid populations, died off within the first few generations, with between 3 and 7 of the original 12 replicates of each treatment surviving for the duration of the experiment (number of surviving beakers = 3 for RP-C, 4 for SD-C, 4 for Hybrid-C, 7 for RP-S, 6 for SD-S, and 4 for Hybrid-S). The proportion of surviving replicates is not significantly different between parental (RP plus SD) and hybrid populations within control conditions (Fisher's exact test, $P = 0.285$), between parental and hybrid populations within salinity stress conditions (Fisher's exact test, $P = 0.144$) or between control and salinity stress conditions (Fisher's exact test, $P = 0.068$). In the surviving beakers, individual survivorship for hybrid replicates in both treatments showed a pattern of heterosis, then hybrid breakdown, followed by recovery to the parental mean (Fig. 1; Table S1a). In the high-salinity treatment, this recovery occurred by generation 9 and was maintained through generation 13. In the control salinity treatment, hybrid replicates did not recover until generation 11 and then dropped below parental fitness again by generation 13.

A second fitness metric, the number of individuals that metamorphosed from larvae to juveniles within 7 days of hatching from eggs, showed a similar pattern, dropping below parental values but then recovering in later generations (Table S1b). Like the survivorship data, the metamorphosis data showed recovery to parental levels by generation 9 in the high-salinity treatment, but not until generation 11 in the control salinity treatment. Unlike the survivorship data, metamorphosis in hybrid replicates at the final time point was equivalent to the midparent in both treatments.

The overall effect of salinity treatment on hybrid fitness in the swarms experiment was tested by repeated-measures ANOVA (Table 1). Results showed no significant effect of treatment (control vs. high salinity), fitness metric (survivorship proportion vs. metamorphosis proportion) or the interaction between generation number and fitness metric. Results did show significant effects of generation and interactions between (treatment and generation), (treatment and fitness metric) and (treatment, generation and fitness metric).

After 14 generations, competitive fitness assays were conducted to provide a more comprehensive measure of fitness. Results showed that samples from hybrid replicates were equivalent to the midparent for the control salinity treatment (Fig. 2a) but greatly exceeded the midparent for the high-salinity treatment (Fig. 2b). Offspring of generation 13 females were also tested for tolerance to a novel stressor, heat shock, by measuring juvenile survivorship under thermal stress. Here, hybrid tolerance was significantly below parentals for the

Table 1 Repeated-measures ANOVA for proportional deviation from the midparent, assessing the effect of treatment (control vs. high salinity), with generation (1,2,3,5,7,9, 11 and 13) and fitness metric (survival proportion and metamorphosis proportion) as repeated measures. *P* values < 0.05 are highlighted in bold.

Source	MS	d.f.	*F*	*P*
Treatment	49 590 004	1	3.504	0.063
Generation	49 603 756	7	3.504	**0.001**
Fitness metric	640 492	1	3.494	0.064
Treatment × Generation	49 601 680	7	3.504	**0.001**
Treatment × Fitness metric	642 758	1	3.506	0.063
Generation × Fitness metric	641 730	7	3.501	**0.001**
Treatment × Generation × Fitness metric	642 457	7	3.505	**0.001**

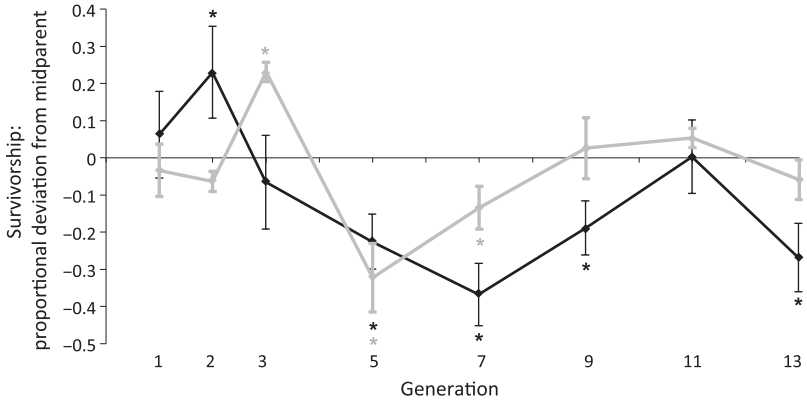

Fig. 1 Mean proportional deviation from midparent among replicates (± 1 standard error) for hybrid survivorship over 13 discrete generations in control salinity (C, black line) and high salinity (S, grey line). Asterisks indicate means significantly different from the midparent according to planned linear contrasts ($P < 0.05$). An average of 19.9 clutches was assayed per treatment per generation; 4787 clutches total.

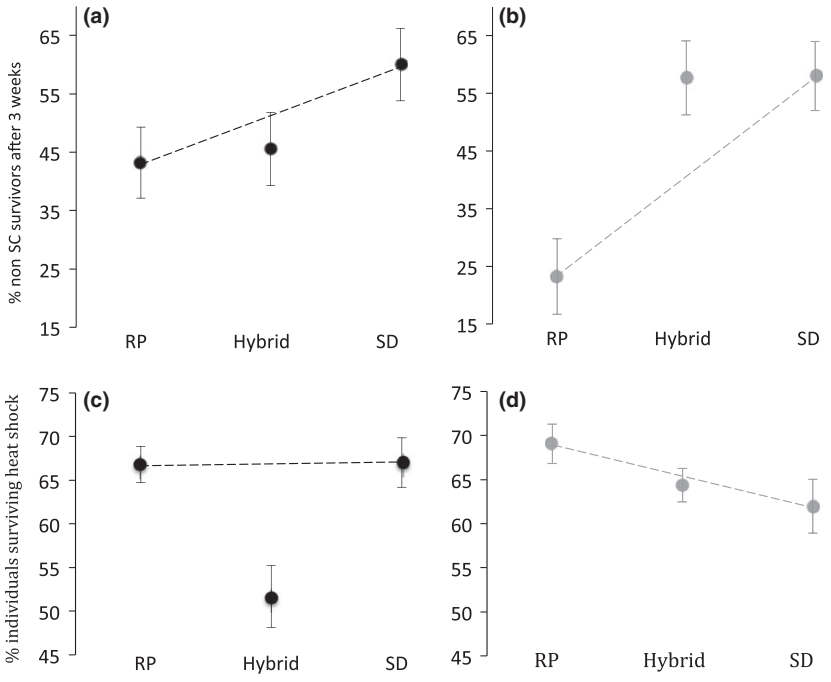

Fig. 2 Generation 14 assays for competitive fitness relative to population SC (a, control salinity, black; b, high salinity, grey; mean of 27.5 assays per population × salinity treatment) and heat-shock survival (c, control salinity, black; d, high salinity, grey; mean of 172.0 assays per population × salinity treatment). Asterisks indicate hybrid means significantly different from the midparent according to planned linear contrasts ($P < 0.05$).

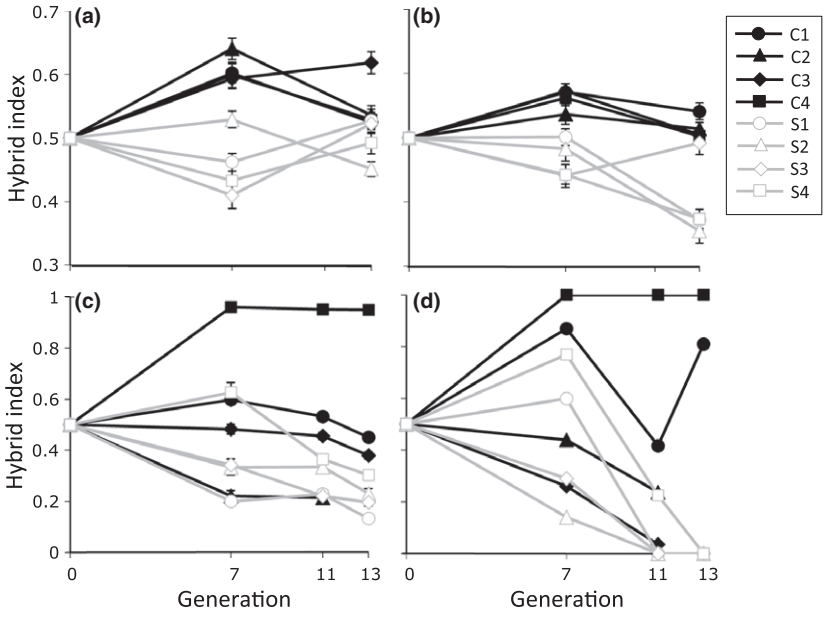

Fig. 3 Mean hybrid indices (± 1 standard error) vs. sampling generation for hybrid replicates in control salinity (C1–C4, black) and high salinity (S1–S4, grey). A hybrid index of 0 indicates 100% RP type whereas an index of 1 indicates 100% SD type. (a) male morphometrics; (b) female morphometrics; (c) nuclear DNA, (d) mitochondrial DNA. In generation 13, there are no molecular data for replicate C2 (small population size) and no mitochondrial data for replicate C3 (insufficient template). Error bars (± 1 standard error) are shown for all panels except d (error bars are not possible here as this is a single locus).

control treatment (Fig. 2c) but equivalent to parentals for the high-salinity treatment (Fig. 2d).

We predicted greater repeatability among hybrid replicates for all metrics due to stronger selection. Results showed greater repeatability for survivorship, but not for morphometrics. For survivorship, one-way ANOVA was performed among hybrid replicates for each treatment and generation. Replicate effects were significant for all eight tested generations in the control salinity treatment, but not for three of the generations (2, 3 and 11) in the high-salinity treatment. Mean variance among replicates, averaged across generations, was significantly greater in the control salinity treatment (0.04) than in the high-salinity treatment (0.01; $P = 0.007$, two-tailed t-test). Greater repeatability in high salinity can also be seen in the trajectories of survivorship for individual replicate beakers (Table S1a).

Morphometrics (Fig. 3, Table S2) based on six characters in females (Fig. 3a) and eight characters in males (Fig. 3b) had similar repeatability among replicates in the two salinity treatments. ANOVA of mean morphometric hybrid index detected a significant replicate effect in almost all generations of both sexes at both salinities. When the variance of hybrid index among hybrid replicates of the same salinity was averaged over all generations, there was no statistically significant difference between treatments in either sex. Morphology of high-salinity replicates tended to favour the RP population, whereas control salinity replicates favoured the SD population.

Genotypic data

Short-term effective population size (N_e) was estimated from the change in microsatellite allele frequencies (Appendix S1) between generations 7 and 13 and was found to be extremely low in all surviving replicates, ranging from 17 to 63 (Table 2). Despite low N_e, molecular composition (Appendix S1) showed repeatable changes among replicates, particularly in the high-salinity treatment. Both treatments showed introgression in early generations, with swamping (particularly for mitochondrial loci) in later generations for a subset of replicates (Fig. 3c,d). *U*-tests across loci showed significant heterozygote deficits in a subset of replicates in both treatments (generation 7: C1, S2; generation 11: C1, C2, C3, S1, S2, S3 and S4; generation 14: C4). In the high-salinity (S) treatment, hybrid indices were more concordant among replicates (Fig. 3c,d), consistently favouring the RP population. Similarly, pairwise F_{ST} values were lower under high salinity (Table S3), with values at the final time point (generation 13) ranging from 0.14 to 0.51 at control salinity and 0.03 to 0.13 at high salinity. Analysis of molecular variance (AMOVA) also showed greater similarity among high-salinity replicates (Table S4). By the final time point, variation among groups was maximized when each control replicate was grouped separately and all high-salinity replicates were grouped together [e.g. (C1) (C3) (C4) (S1, S2, S3 and S4)].

Of particular interest is whether selection on hybrid gene interactions differed between salinity treatments. Two-locus comparisons showed stronger maintenance of parental genotypes in the more stressful environment. We limit our mitonuclear comparisons to generation 7, as replicates in later generations frequently became swamped for single mitochondrial haplotypes (e.g. Fig. 3d). In generation 7, 1 of 3 control (C) replicates and 3 of 3 salinity stress (S) replicates showed a significant excess of matched mitonuclear genotypes, where homozygous nuclear genotypes and mtDNA haplotypes come from the same population, and a significant deficit of unmatched genotypes, where mtDNA and

Table 2 Estimated effective population size (N_e) in hybrid control replicates (C) and high-salinity replicates (S) obtained using the standard variance in allele frequency change (F) according to the temporal method of Nb_HetEx (Zhdanova & Pudovkin, 2008).

Replicate	F	N_e (F)	95% CI
C1	0.1284	29.2	14.9–52.7
C3	0.1590	22.5	12.1–38.2
C4	0.0738	63.1	22.9–182.6
S1	0.1250	30.6	13.9–60.8
S2	0.1074	37.5	17.5–75.4
S3	0.1271	29.8	16.5–50.6
S4	0.2021	17.0	9.2–28.6

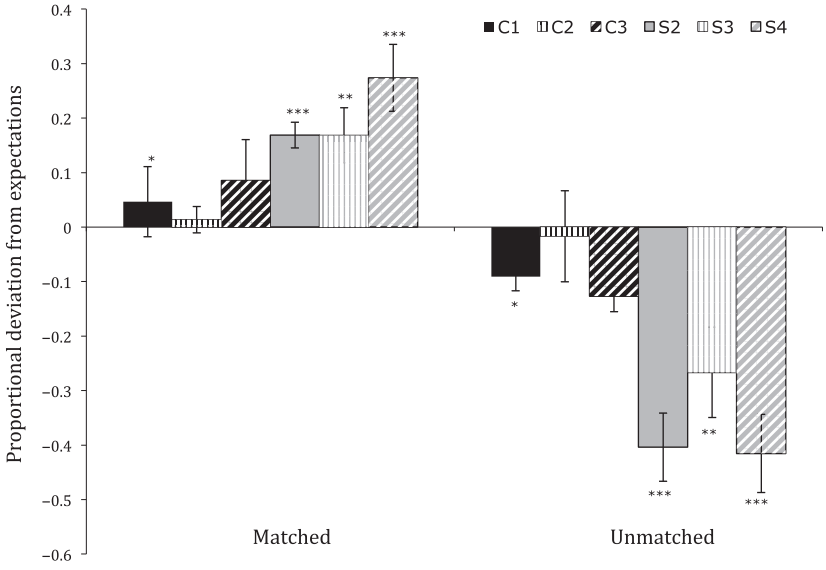

Fig. 4 Mean (± 1 standard error) for proportional deviation from expected two-locus mitonuclear genotype frequencies at generation 7. Matched individuals have mtDNA and homozygous nuclear genotypes from the same population. Unmatched individuals have mtDNA and homozygous nuclear genotypes from different populations. Asterisks indicate the significance of one-tailed *t*-tests of observed vs. expected genotype numbers (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). An average of 36.4 individuals was genotyped per replicate per two-locus combination. Replicate C4 was excluded due to mtDNA fixation, and replicate S1 was excluded due to a high frequency of missing mtDNA haplotypes.

homozygous nuclear genotypes come from different populations (Fig. 4). The same pattern was not as apparent for nuclear–nuclear comparisons (Fig. S3). Here, both treatments show similar maintenance of coadapted parental genotypes in generation 7, with a general excess of matched homozygotes and deficit of unmatched homozygotes. However, pairwise tests of linkage disequilibrium between loci on different chromosomes did find more significant interactions (post-Bonferroni corrections) within replicates in the high-salinity treatment (16, 0 and 0 significant pairs for generations 7, 11 and 13) than in the control treatment (2, 1 and 0 significant pairs), but only for the earliest time point.

Discussion

Consequences of hybridization

In both the control and high-salinity treatments, the temporal trajectory of hybrid fitness showed a trend towards increase, then a significant decline, and then recovery to parental values. This ultimate recovery is consistent with previous studies of the species showing that long-term consequences of interpopulation hybridization are generally benign, even for pairs of populations exhibiting substantial fitness problems in early generations (Hwang *et al.*, 2011, 2012; Pritchard & Edmands, 2013; Pritchard *et al.*, 2013).

In the current study, the recovery of fitness across independent replicates is perhaps surprising in view of the low N_e estimates for these replicates (17–63). The low effective population sizes of these experimental replicates may not be atypical of those in natural tide pool populations, which are believed to experience frequent bottlenecks (Dybdahl, 1994; Burton, 1997; Edmands & Harrison, 2003). Fitness recovery is also somewhat surprising given the high level of genetic divergence between the tested populations. Despite high genetic differentiation and the predominate force of genetic drift, the repeated recovery of fitness in our experimental hybrid populations implies efficient selection on recombinant genotypes, perhaps aided by the relative simplicity and consistency of laboratory conditions.

Consequences of stress

The high-salinity treatment of 53 ppt is well within the range of salinities (6–102 ppt) that *T. californicus* tolerates in nature (Egloff, 1967; Burton *et al.*, 1979), and yet both the pilot study and the swarms experiment showed this treatment to be stressful. This stressful treatment promoted faster recovery from hybrid breakdown, with both survivorship and metamorphosis values returning to parental levels up to two generations earlier than in the control. This faster recovery under salinity stress is confirmed by the significant treatment by generation interaction in the repeated-measures ANOVA. Stress also promoted a stronger recovery, as stressed hybrids had survivorship equivalent to the midparent through the final time point whereas control hybrids dropped back below the midparent. Similarly, stressed hybrids from the final time point performed better than control hybrids in both the competitive fitness and heat-shock assays. Finally, stress promoted a more repeatable recovery from hybridization, as seen in results for survivorship and molecular composition.

The finding that stressed hybrids experience stronger and more repeatable recovery, despite equivalent effective population size (N_e), implies more efficient selection (N_eS), with stressful conditions increasing the intensity of selection (S). Interestingly, the high-salinity treatment favoured RP alleles and morphology even though the RP population was found to be competitively inferior to the SD population at both salinities. It may be that only a subset of RP alleles are advantageous at high salinity, or that RP alleles are particularly favoured on a hybrid background. Also interesting is that hybrids raised under salinity stress performed better under thermal stress than hybrids raised in benign conditions, suggesting there may be overlap in the mechanism of tolerance to these different stressors. In the swarms experiment, the majority of replicates died out in the early generations, regardless of whether they were hybrid or parental populations and whether they were in control or high-salinity conditions. This high mortality in the early generation of lab culture is consistent without previous similar experiments (Hwang *et al.*, 2011, 2012; Pritchard & Edmands, 2013; Pritchard *et al.*, 2013), although the cause remains unknown.

In some cases, hybrids may be favoured in novel or stressful habitats because they express trait values that fall outside the range of both parentals, a phenomenon known as transgressive segregation (Slatkin & Lande, 1994; Rieseberg *et al.*, 1999; Pereira *et al.*, 2014; Stelkens *et al.*, 2014; Hamilton & Miller, 2016). We did not detect hybrid transgression for survivorship or metamorphosis. We did, however, detect hybrid transgression for morphometric characters, as has been shown in hybrid swarms between different populations of *T. californicus* (Pritchard *et al.*, 2013). In the current study, these examples of morphometric transgression did not persist across generations. Further, there was no evidence for transgressive hybrid morphology being more favoured under salinity stress than under control conditions. It may be that the specific characters measured were not under strong selection, or that the size of the study and magnitude of genetic drift prevented detection of such patterns.

Comparisons to previous studies

While previous experimental work has not addressed long-term consequences of interpopulation hybridization with and without environmental stress, there have been

a number of multigeneration hybridization studies under benign conditions. The current study is most similar to that of Hwang *et al.*, 2011; who assessed long-term consequences of hybridization in the same two *T. californicus* populations over a period of 15 months. The current study differs from this previous work in that discrete (rather than overlapping) generations were maintained, and genomewide markers were assayed. Looking only at results under control conditions, both the current study and Hwang *et al.* (2011)'s study found that the fitness of hybrid replicates dropped below midparent values in early generations and then recovered. However, recovery was stronger in this previous experiment, with hybrid fitness ultimately exceeding both parentals instead of only one. The creation of superior hybrids in this previous experiment may have been facilitated by the protocol of maintaining overlapping generations, which would allow the fittest recombinant genotypes to persist and reproduce over multiple generations. Maintaining discrete generations is more time-consuming than maintaining overlapping generations, but has the distinct advantage of allowing consequences of hybridization to be tracked each generation.

In other taxa, the few cases in which hybridization has been monitored across many generations demonstrate that this pattern of hybrid fitness problems in early generations followed by recovery in later generations is not universal. Intraspecific hybridization between inbred lines of *Drosophila* (Bijlsma *et al.*, 2010; Pekkala *et al.*, 2012) resulted in viability levels equivalent or higher than parentals for all tested generations (up to generation 10). Interspecific hybridization in *Drosophila* (Hercus & Hoffman, 1999) resulted in viability levels equivalent to the superior parent at generations 17 and 30, although effects in earlier generations were not measured. In the legume *Chamaecrista fasciculata*, crosses between populations known to exhibit hybrid breakdown showed an increase and full recovery of fitness between the F2 and F6 generations, suggesting selection on beneficial recombinant genotypes (Erickson & Fenster, 2006). Finally, hybridization between wild populations of two species of *Ambystoma* salamanders resulted in fitness problems that persisted even after approximately 20 generations of admixture (Johnson *et al.*, 2010).

Targets of selection

The repeatable success of hybrids in the high-salinity treatment suggests more efficient selection in more stressful conditions. The very modest number of loci surveyed in this study does not allow identification of particular candidate loci under selection, but we can assess overall patterns of between-locus interactions. Two-locus comparisons show that salinity stress favours an excess of matched mitonuclear combinations but not nuclear–nuclear combinations, suggesting stronger selection for maintaining mitonuclear coadaptation in the high-salinity environment. This is consistent with a previous study of the species (Ellison & Burton, 2008), which found reduced transcriptional response to osmotic stress in mismatched mitonuclear hybrids and implicated interactions between mtDNA and nuclear-encoded mitochondrial RNA polymerase. Beyond *Tigriopus*, work on other species shows mitonuclear interactions to be pervasive (Rand *et al.*, 2004; Burton & Barreto, 2012; Wolff *et al.*, 2014), and often modified by stressors such as temperature (Dowling *et al.*, 2007) and dietary restriction (Zhu *et al.*, 2014).

Implications for conservation and evolution

In a conservation context, our results support the optimistic prediction that intentional translocations aimed at genetic rescue (Hedrick & Fredrickson, 2010; Heber *et al.*, 2013) may increase fitness in the long run, if populations can survive any fitness problems that may occur in early generations. Perhaps counter-intuitively, our study suggests the ultimate likelihood of success may be higher under more environmentally challenging conditions. On the negative side, accidental mixing of populations or species may be particularly likely to allow hybrid introgression under stressful conditions, potentially displacing natives and disrupting communities (Ellstrand & Schierenbeck, 2000; Ryan *et al.*, 2009, 2013). Our results are consistent with the observed association between hybrid speciation and stressful habitats (Nolte *et al.*, 2005; Arias *et al.*, 2008; Donovan *et al.*, 2010), providing experimental evidence for a reduction in the deleterious effects of hybridization under stressful conditions.

Acknowledgments

For assistance with culture maintenance and molecular assays, we thank M. Chinen, A. De la Torre, K. Liu, P. Luong, A. Myrie, S. Roen-Padilla, N. Sargsyan, H. Truong & Z. Salahuddin. Funding was provided by U.S. National Science Foundation grants DEB-0709775 (A.H. and S.E.), DEB-0316807 (S.E.) and DEB-1355170 (S.E.).

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1 Genotype data in Genepop format.

Table S1 Mean survivorship and metamorphosis values in each generation and treatment, relative to midparent values.

Table S2 Mean morphometric values for males and females in each generation and treatment.

Table S3 Pairwise F_{ST} between replicates in generations 7 and 13.

Table S4 Analysis of molecular variance showing the grouping of replicates that maximized among group variance.

Figure S1 Pilot test of growth rate at different salinities.

Figure S2 Proportion of clutches with larvae still present at day 7.

Figure S3 Deviation from expected frequencies for four two-locus genotype classes.

Data deposited at Dryad: 10.5061/dryad.p3p4p

Received 16 October 2015; accepted 30 May 2016