



ECOLOGICAL NOVELTY BY HYBRIDIZATION: EXPERIMENTAL EVIDENCE FOR INCREASED THERMAL TOLERANCE BY TRANSGRESSIVE SEGREGATION IN *TIGRIOPUS CALIFORNICUS*

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Early generations of hybrids can express both genetic incompatibilities and phenotypic novelty. Insights into whether these conflicting interactions between intrinsic and extrinsic selection persist after a few generations of recombination require experimental studies. To address this question, we use interpopulation crosses and recombinant inbred lines (RILs) of the copepod *Tigriopus californicus*, and focus on two traits that are relevant for the diversification of this species: survivorship during development and tolerance to thermal stress. Experimental crosses between two population pairs show that most RILs between two heat-tolerant populations show enhanced tolerance to temperatures that are lethal to the respective parentals, whereas RILs between a heat-tolerant and a heat-sensitive population are intermediate. Although interpopulation crosses are affected by intrinsic selection at early generational hybrids, most of the sampled F₉ RILs have recovered fitness to the level of their parentals. Together, these results suggest that a few generations of recombination allows for an independent segregation of the genes underlying thermal tolerance and cytonuclear incompatibilities, permitting certain recombinant lineages to survive in niches previously unused by parental taxa (i.e., warmer thermal environments) without incurring intrinsic selection.

KEY WORDS: Adaptation, hybridization, reproductive isolation, speciation.

The evolutionary significance of hybridization has historically been a topic of strong disagreement among evolutionary biologists (Harrison 1993, 2012; Mallet 2008a,b). Research on the genetics of intrinsic postzygotic isolation has led many researchers to conclude that the manifestation of genetic incompatibilities in hybrids demonstrates that isolating barriers between species are byproducts of divergence in allopatry (Muller 1942; Orr 1995). This resulted in a generalized view, particularly among zoologists, that species formation relies on the evolution of these intrinsic isolating mechanisms and that hybridization is rarely a source of evolutionary novelty. Thus, hybridization would lead almost exclusively either to the merging of parental taxa or to

the reinforcement of isolation mechanisms following secondary contact (Liou and Price 1994). In contrast, other authors advocate that hybridization may provide the raw material for rapid adaptation (Anderson and Stebbins 1954; Stebbins 1959; Lewontin and Birch 1966; Arnold 1997; Grant and Grant 2011), and that it provides a simple explanation for niche divergence and phenotypic novelty often associated with hybrid lineages (Stebbins 1959; Grant 1981; Abbott 1992; Rieseberg 1997). Although both postzygotic isolation and phenotypic novelty are not exclusive outcomes of hybridization, insights into how different hybrid lineages are impacted by these opposite forces require experimental studies integrating both outcomes of hybridization.

The study of both experimental crosses in the laboratory and natural hybrid zones has shown that hybridization is frequently accompanied by a decrease in intrinsic fitness—that is, independent of the habitat (Barton and Hewitt 1985). This recurring pattern of hybrid sterility or inviability (partial or complete) is a confirmation of the Dobzhansky–Muller model, which posits that postzygotic isolation arises in allopatry as a side-effect of stochastic evolutionary divergence. These incompatibilities result from the coevolution of different genes interacting within the same genomic environment and involve both interactions among nuclear genes (Presgraves 2010) and cytonuclear interactions (Rand et al. 2004; Burton and Barreto 2012). Thus, intrinsic selection on hybrid genotypes poses an important challenge for populations of hybrid origin, particularly when competing with their parental taxa.

However, extrinsic selection—that is, dependent on the habitat—might impact hybridization either negatively or positively. Theoretical and empirical studies (Lewontin and Birch 1966; Grant 1981; Buerkle et al. 2000; Lexer et al. 2003) suggest that hybrid populations are likely to persist only if they can occupy previously underused fitness peaks on the local adaptive landscape. However, in most cases hybrid phenotypes are intermediate and thus, without niche or geographic separation, new hybrid genotypes are likely to be overcome by competition and/or gene flow from parental populations (Arnold 1997; Rieseberg 1997). Alternatively, hybridization can generate phenotypes that are extreme relative to those of either parental (deVicente and Tanksley 1993; Rieseberg et al. 1993; Cossé et al. 1995)—a mechanism referred to as transgressive segregation. The genetic basis of transgressive segregation is largely distinct from that underlying heterosis, because the former is caused by heritable complementary gene action, rather than heterozygosity and dominance effects that are most pronounced in first-generation hybrids. Among all the reported traits exhibiting transgressive segregation, transgression in tolerance to various biotic or abiotic factors (e.g., temperature, salinity, desiccation, toxicity) may be most important to the evolution of populations of hybrid origin, because variation in ecological tolerances seems most likely to facilitate niche divergence (Slatkin and Lande 1994; Rieseberg et al. 1999). Thus, depending on the quantitative traits segregating in hybrid populations, extrinsic selection can either lead to the fusion of parental lineages or to the proliferation of a hybrid lineage.

The copepod species *Tigriopus californicus* provides an ideal system to evaluate the combined impacts of intrinsic and extrinsic selection in hybridization. Geographically isolated populations have diversified along the west coast of North America, from Baja California to Alaska, resulting in a gradient of genetically and ecologically divergent populations (Edmands 2001; Willett 2010). Interpopulation crosses in this species have revealed intrinsic cytonuclear incompatibilities between natural populations

(Edmands and Burton 1999; Rawson and Burton 2002; Ellison and Burton 2008), and that the magnitude of hybrid breakdown is correlated with the genetic differentiation between parental taxa (Edmands 1999). In addition, populations have also locally adapted to the strong extrinsic thermal gradient observed along the species distribution, so that southern populations have higher heat-tolerance than northern populations (Willett 2010). Although the underlying molecular mechanisms involved in adaptive and nonadaptive divergence remains a topic of current research in *T. californicus* (Schoville et al. 2012), they likely have a complex and polygenic basis. Thus, hybridization between these populations can potentially generate a wide range of unique combinations of parental genotypes, with widely varying intrinsic and extrinsic fitnesses. Previous assays of F2 hybrids showed a general trend of decreased intrinsic fitness, or hybrid breakdown (Ellison and Burton 2008), and extrinsic fitness intermediate to parental taxa (Willett 2012a). However, those studies offered a limited view on the real fitness of hybrid genotypes because they were lacking an explicit integration of both intrinsic and extrinsic selective regimes, and relied on fitness averages across many F2 individuals, which are characterized by various recombinant genotypes and high heterozygosity.

In this study, we use the *T. californicus* system to provide insights on two fundamental questions regarding the evolutionary consequences of hybridization: (1) Can hybridization lead to the colonization of new niches by transgressive segregation in ecologically relevant traits?; and (2) When genetic incompatibilities have already evolved between parental taxa, are hybrid genotypes with transgressive phenotypes always characterized by decreased intrinsic fitness? In addition to F1 and F2 hybrids, we use recombinant inbred lines (RILs) characterized by unique mosaic genomes of the parental forms to assess the fitness of unique recombinant genotypes segregating in a hybrid population. We focus both on intrinsic and extrinsic components of fitness by measuring two traits relevant for the diversification within *Tigriopus*, survivorship during early development and tolerance to heat-stress, respectively.

Materials and Methods

POPULATION SAMPLING

Populations of *T. californicus* distributed along coastal California represent much of the breadth of ecologic and genetic divergence known in this species (Edmands 2001; Willett and Ladner 2009; Willett 2010). As a heat-sensitive parental taxon, we chose the northern population of Santa Cruz (herein SC: 36°56'58.32"N, 122°2'48.98"W). As heat-tolerant taxa, we chose the southern populations of Bird Rock (herein BR: 32°48'51.00"N, 117°16'24.08"W) and San Diego (herein SD: 32°44'41.17"N, 117°15'19.43"W; Fig. 1).

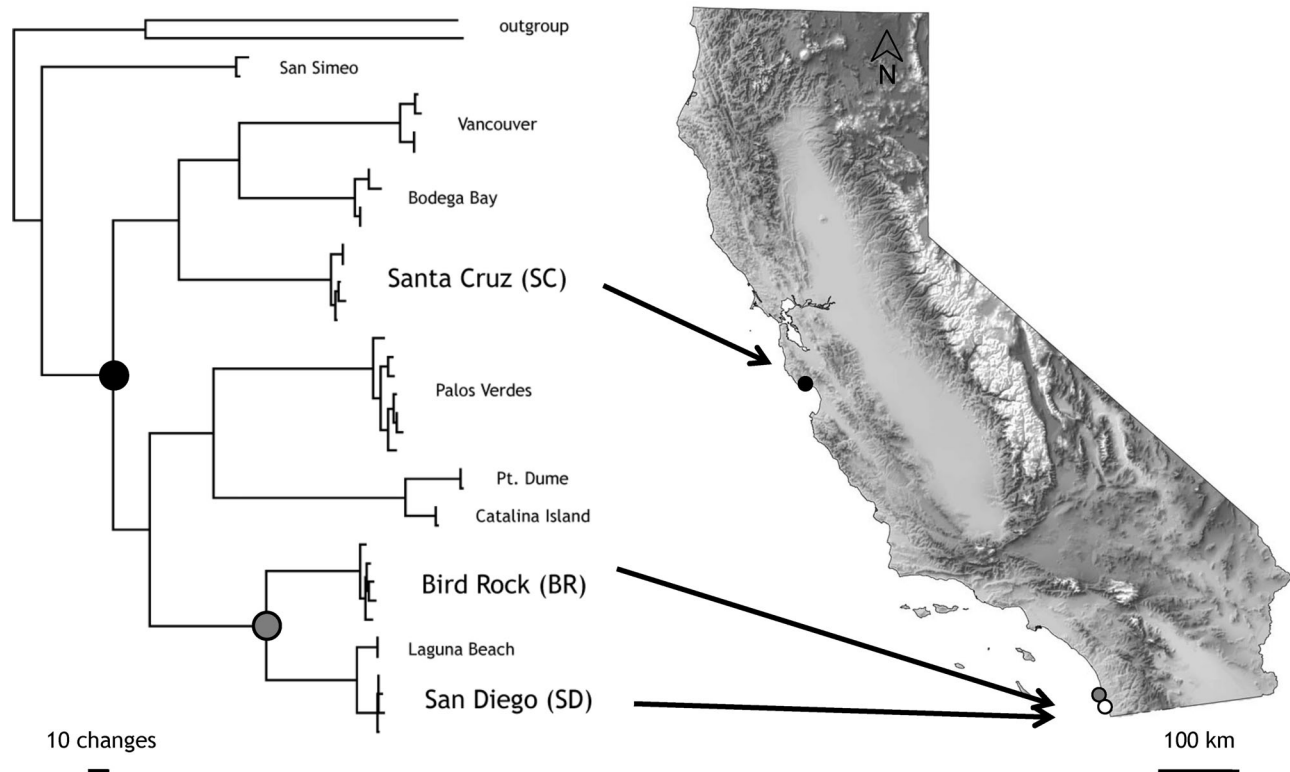


Figure 1. Population sampling for *Tigriopus californicus*. The phylogenetic tree represents the diversification of the species along the west coast of North America, based in mitochondrial *cytb* sequences (Willet and Ladner 2009). Circles at the nodes mark the phylogenetic depth of the two experimental crosses used on this work: the genetically (and ecologically) divergent populations of Santa Cruz and Bird Rock (black), and the genetically (and ecologically) similar populations of Bird Rock and San Diego (gray).

At each locality, copepods were collected from several high intertidal pools and combined into a single stock culture. All cultures were maintained at 20°C with a 12:12 L:D photoperiod, in filtered seawater mixed with ground “Algae Wafers” (Kyorin Co., Himeji, Japan), in 400 mL beakers. Replicated beakers from each population were maintained for at least one generation before the assays, to avoid effects of phenotypic plasticity, and were periodically mixed together to promote panmixia.

LOCAL ADAPTATION BETWEEN PARENTAL POPULATIONS

To measure the extrinsic fitness of different parental populations to heat-stress, we followed the acute temperature stress assay that was previously used to measure the latitudinal gradient of thermal tolerance in natural populations (Willet 2010). Ten males and 10 females from each genotype were placed into a 15 mL Falcon tube with 10 mL of filtered seawater. After an acclimatization period of 1 hour in a beaker of water at 20°C, the tubes were immersed in a water bath at a target stress-inducing temperature for 1 hour. During this assay, the copepods inside the tube are exposed to about 30 min of increasing temperature and 30 min at the target temperature. Following stress, tubes were immersed in 20°C water

for a 1-hour period of recovery; copepods were then transferred to culture dishes with food at 20°C. Survivorship was scored separately for males and females as the fraction of live animals after 3 days. We pooled males and female replicates together, after testing for differences in survivorship between sexes at each temperature. To test for differences in heat tolerance between parental taxa, we performed the assay between 33°C and 37°C, with at least 16 replicates at each target temperature. Replicates from different population were assayed at the same time, so that genetic differences are not confounded by temporal differences of the assay.

Survivorship to heat-stress is not expected to follow a normal distribution, and thus nonparametric tests are the most appropriate to assess statistical significance. For each temperature, we tested for differences across all taxa using a Kruskal–Wallis rank sum test. We tested for differences between populations using a Mann–Whitney *U*-test for all pairwise comparisons, followed by a false discovery rate (FDR) adjustment of the *P*-values. All statistical analyses were performed in R 2.15.1 (R Development Core Team). Based on studies in other populations (Willet 2010), we expect that the northern population of SC will be less tolerant to acute heat-stress than the southern populations of BR and SD.

EXPERIMENTAL HYBRIDIZATION

In addition to the differential heat tolerance mentioned earlier, previous genetic work showed that these populations have contrasting degrees of genetic divergence (Willett and Ladner 2009). The SC and BR populations are more genetically divergent (20.2% sequence divergence in *cytb*) than the BR and SD populations (10.4% sequence divergence in *cytb*). Although the magnitude of mitochondrial divergence within *Tigriopus* is notably higher than in many other organisms, sequence divergence of mitochondrial genes such as *cytb* should be regarded as a relative measure of divergence, because the mtDNA of *Tigriopus* is estimated to evolve approximately 55 times faster than nuclear DNA (Willett 2012b). We hence performed crosses between the two populations that have highly diverged both genetically and ecologically (BR × SC), and between the two populations that are genetically and ecologically more similar (BR × SD).

Tigriopus californicus females mate only once, and mature males clasp virgin females with their antennae until the females are reproductively mature (Burton 1985). In our experimental crosses, virgin females were separated from clasped males using a fine needle, then mated with males from the required genotype in a culture dish. For all crosses, adult males were removed from the cultures when females developed egg sacs and adult females were removed at the first appearance of copepodid (juvenile copepod) stage individuals, to maintain nonoverlapping generations. To generate F2 and F3 hybrids, we used individuals from replicated culture dishes, assuring a no-inbreeding strategy through the first three generations of hybridization. This strategy results in F3 outbred individuals, with various recombinant genotypes and high heterozygosity.

To assess the fitness of each recombinant genotype without the confounding effect of heterozygosity, we allowed for five further generations of full-sib matings (full-sib matings reduce heterozygosity by 19% per generation; Hedrick 2011). As F3 females with egg sacs appeared, we individually isolated approximately 40 females to start unique RILs. Thus, in total we generated at least 160 hybrid lines for the two interpopulation and reciprocal crosses. Discrete generations were maintained up to the F9 generation; at that point, each isofemale line was maintained in mass culture with overlapping generations. This strategy results in F9 RILs with known (matrilineally transmitted) mitochondrial background and unique recombinant nuclear genotypes with ~40% of their original heterozygosity. To distinguish between hybrid genotypes interacting with different mitochondrial lineages, each cross is coded by its maternal followed by its paternal ancestry (e.g., BR × SC F1 has the BR mtDNA whereas its reciprocal cross SC × BR F1 has the SC mtDNA).

The number of RILs that reach F9 generation is expected to be a reduced subset of the initial number of isofemales lines. Moreover, this approach may select for lines with higher survivorship

(i.e., lines carrying alleles causing outbreeding and inbreeding depression will tend to die off before reaching F9 generation). To measure the effect of our experimental hybridization approach in the number of surviving RILs and their fitness, we established approximately 30 parental inbred lines for each population.

EXTRINSIC SELECTION DURING HYBRIDIZATION

To test for differences in heat tolerance between parental and recombinant genotypes, we performed the assay described earlier at a sublethal temperature—the lowest temperature at which one or both parentals have ~20% survivorship—and at a lethal temperature—the lowest temperature at which one or both parentals do not survive. Because interpopulation hybrids in copepods typically show hybrid breakdown (Burton et al. 2006), the number of replicates varied among population crosses, generation of hybridization, and mitochondrial lineage. For F1, F2, and inbred F9 hybrids, we were able to replicate the assay respectively 8, 6, and 4 times.

We used mean parental survivorship (± 1 SE) to define “parental fitness.” Mean survivorship of hybrid genotypes above “parental fitness” is suggestive of “hybrid vigor,” whereas a lower mean is suggestive of “hybrid breakdown.” Similarly to the comparisons among parental populations, we tested for overall differences in fitness using a Kruskal–Wallis rank sum test. We also tested for significant hybrid vigor in heat tolerance using a Mann–Whitney *U*-test to compare hybrids with putative hybrid vigor to the most heat-tolerant parental population. When appropriate, *P*-values were corrected for multiple comparisons using an FDR adjustment.

INTRINSIC SELECTION DURING HYBRIDIZATION

Interpopulation hybridization in *T. californicus* typically results in breakdown in three life-history traits: fecundity, survivorship, and metamorphosis (Edmands 1999; Burton et al. 2006); but crosses with the BR population have not yet been tested. We measured the effect of intrinsic selection in parentals, F1, F2, and F9 RILs as reflected by survivorship, because our preliminary data showed a stronger variance among genotypes at this trait than at the other two. Gravid females carrying eggs with the genotype of interest were isolated in clean culture dishes and monitored. All the newly hatched nauplii were transferred to a new dish. Survivorship was calculated as the percentage of nauplii surviving to 14 days. The assay was replicated at least 10 times for each genotype.

For each cross, mean parental survivorship (± 1 SE) was used to define “parental fitness,” “hybrid vigor,” and “hybrid breakdown” as described earlier. Intrinsic hybrid breakdown is expected to result in nonnormal distributions of survivorship, and thus nonparametric methods are the most adequate to assess statistical significance. We tested for overall differences in fitness using a Kruskal–Wallis rank sum test. We then tested for

significant hybrid breakdown using a Mann–Whitney *U*-test to compare hybrids with putative hybrid breakdown to the parental population with the lowest fitness. We used an FDR adjustment to correct *P*-values for multiple comparisons. In the presence of significant hybrid breakdown, we also tested for asymmetries between reciprocal crosses that only differ in their mitochondrial DNA. Based on studies with other populations (Edmands 1999), we predict that F1 hybrids will show hybrid vigor due to heterosis, whereas F2 and inbred lines will show hybrid breakdown due to recombination. The magnitude of vigor and breakdown should depend on the degree of genetic divergence between parental taxa.

IMPACT OF EXTRINSIC AND INTRINSIC SELECTION IN DIFFERENT RILS

Several studies have shown that interpopulational hybrids of *T. californicus* are affected by intrinsic selection (Burton et al. 2006), extrinsic selection (Willett 2010), and by an interaction among these two (Rawson and Burton 2002; Willett and Burton 2003). Yet, it remains unknown whether loci underlying both selective regimes are physically linked, or whether a few generations of recombination may lead to an independent segregation of the loci underlying extrinsic and intrinsic selection. To address this question we focus on the F9 RILs, which should each reflect a unique mosaic of the genetic variation that arises at F3 generation by recombination, but with increased homozygosity.

Both intrinsic and extrinsic fitness of each recombinant line were standardized by subtracting the respective midparent mean. Thus, positive values of fitness would indicate higher fitness relative to parental taxa, whereas negative values would have the opposite effect. The relative extrinsic fitness was considered only for the stress-inducing temperature tested in both crosses (35°C).

Results

LOCAL ADAPTATION BETWEEN PARENTAL POPULATIONS

We did not observe significant differences in survivorship between sexes at any temperature (Mann–Whitney *U*-test; all *P* > 0.13). Thus, for each genotype, assays with males and females were considered independent replicates (Table S1).

The tolerance to heat-stress was higher in the southern populations (Fig. 2). As the temperature of the heat-stress increases to 34°C, populations differ significantly in survivorship ($\chi^2 = 18.5$; *df* = 2; *P* < 0.001), with the northern population (SC) having lower survivorship than both southern populations (Mann–Whitney *U*-test; all *P* < 0.003). At 35°C survivorship remains different across populations ($\chi^2 = 14.3$; *df* = 2; *P* < 0.001), with SC reaching its lethal temperature and being significantly different from BR and SD (Mann–Whitney *U*-test; both *P* < 0.002).

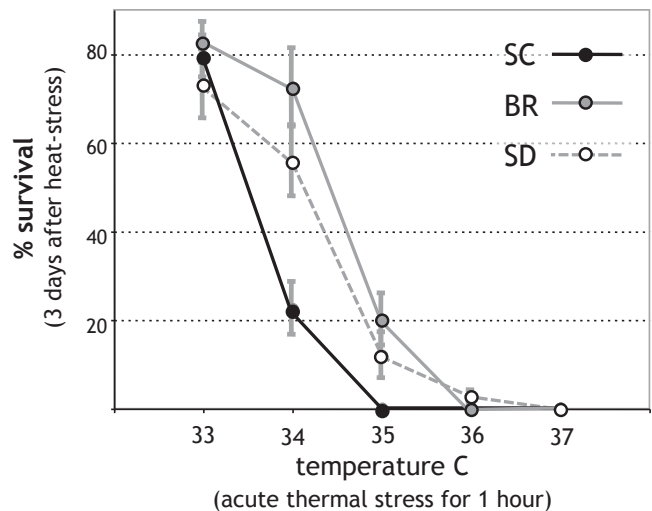


Figure 2. Different heat tolerance in parental taxa. Lines connect mean survivorship for putative heat-sensitive (black) and heat-tolerant (gray) populations; error bars are ± 1 SE.

Survivorship did not differ significantly at 36°C, with this being a lethal temperature for all three populations. Statistical significance levels for all pairwise comparisons are listed in Table S2.

EXPERIMENTAL HYBRIDIZATION

The majority of isofemale lines did not survive to F9 generation, both in the recombinant and in the parental inbred lines. The number of RILs that survived to F9 generation is higher in crosses between ecologically and genetically similar populations (BR \times SC-5; BR \times SD-13). Also, in both crosses, more RILs carrying BR mtDNA survived to F9 than did those carrying mtDNA from either SC or SD (BR \times SC-4:1; BR \times SD-12:1). None of these biases are statistically significant and thus a random extinction of lineages cannot be rejected. We observe a similar decrease in the control parental inbred lines that survived to F9 generation (BR \times BR-1; SD \times SD-3; SC \times SC-4).

Among the surviving lines, not all were equally productive or had a balanced sex ratio. Thus, the number of assays for extrinsic and intrinsic fitness in a few cases varied across genotypes, temperatures, or sexes (Tables S1, S3).

EXTRINSIC SELECTION DURING HYBRIDIZATION

Because parental populations have different sublethal and lethal temperatures, hybrids between heat-tolerant and heat-sensitive taxa (BR \times SC) were tested at 34°C and 35°C, whereas hybrids between heat-tolerant taxa (BR \times SD) were tested at 35°C and 36°C (Fig. 3). Although for every temperature and cross we always observe differences in survivorship to heat-stress (Kruskal–Wallis rank sum test; all *P* < 0.002), the fraction of hybrid genotypes with increased tolerance varied widely between crosses.

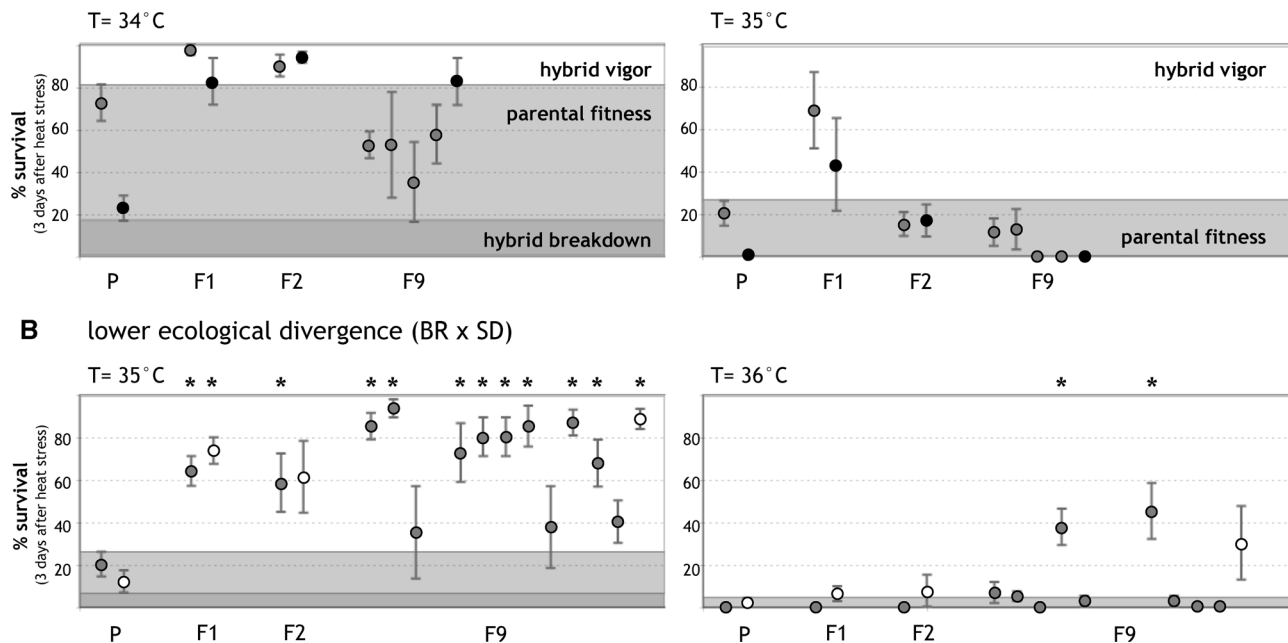
A higher ecological divergence (BR x SC)


Figure 3. Extrinsic hybrid fitness at different levels of ecological divergence. The graphics refer to survivorship to heat-stress (mean \pm 1 SE) in crosses between taxa with high (A) and low (B) ecological divergence. Each genotype is colored accordingly to its mitochondrion (SC: black; BR: gray; SD: white). The left panels show survivorship to temperatures near the physiological limit of the parental taxa, whereas right panels show survivorship at a lethal temperature to one or both parental taxa. “Parental fitness” is defined by the mean survivorship (\pm 1 SE) of parental taxa. Thus, higher survivorship is suggestive of “hybrid vigor” whereas lower survivorship is suggestive of “hybrid breakdown.” Hybrid genotypes that are characterized by significant hybrid vigor in heat tolerance are demarked with * (Mann–Whitney *U*-test; all *P*, 0.016).

Between BR \times SC (Fig. 3A), at both temperatures, F1 hybrids have higher mean survivorship to heat-stress than parentals (putative hybrid vigor), and at the lower temperature (34°C) the same is observed at F2 hybrids and one RIL. However, none of these differences is significant.

In contrast, the evidence for hybrid vigor in relation to heat-stress is more prevalent in crosses between BR \times SD (Fig. 3B). When parentals are at their sublethal temperature (35°C), all hybrids have higher mean survivorship. From these, both F1 hybrids, F2 hybrids with BR mtDNA, and 7 inbred lines have significantly higher survivorship than the most heat-tolerant parental (Mann–Whitney *U*-test; all *P* < 0.05). Remarkably, when these genotypes are exposed to a temperature that is lethal to either parental (36°C), five RILs have higher mean survivorship than the most tolerant parental, two of which with statistical significant differences (Mann–Whitney *U*-test; all *P* < 0.002). Statistical results for all comparisons are listed in Table S2.

INTRINSIC SELECTION DURING HYBRIDIZATION

Both crosses resulted in significant differences in intrinsic fitness (Fig. 4; overall Kruskal–Wallis rank sum test, *P* < 0.017), and

showed F1 hybrids and some RILs with mean fitness lower than parentals.

In crosses between BR \times SC, we observed a fitness decrease in the F1 with SC mtDNA and in two RILs, suggestive of hybrid breakdown (Fig. 4A). Although hybrid breakdown at the F1 with SC mtDNA is marginally significant when compared to the parental with lowest fitness (Mann–Whitney *U*-test; *P* = 0.042), when we consider the multiple comparisons with the RILs with putative hybrid breakdown and adjust the *P*-value, this comparison becomes not significant. Hybrid breakdown in the RILs is not significant. Intrinsic fitness of F1 hybrids is highly asymmetric, with the F1 carrying the SC mtDNA being significantly distinct from the reciprocal F1 with BR mtDNA (Mann–Whitney *U*-test; *P* = 0.014).

In crosses between BR \times SD, we observe some decrease of intrinsic fitness, but at a lower magnitude than in the previous cross (Fig. 4B). Again, F1 hybrids show lower survivorship than parentals, but without apparent asymmetries between mitochondrial lineages. Although hybrid breakdown is marginally significant for F1 hybrids with BR mtDNA (Mann–Whitney *U*-test; *P* = 0.038), this difference is not significant after *P*-values are adjusted for multiple comparisons. Hybrid breakdown in the

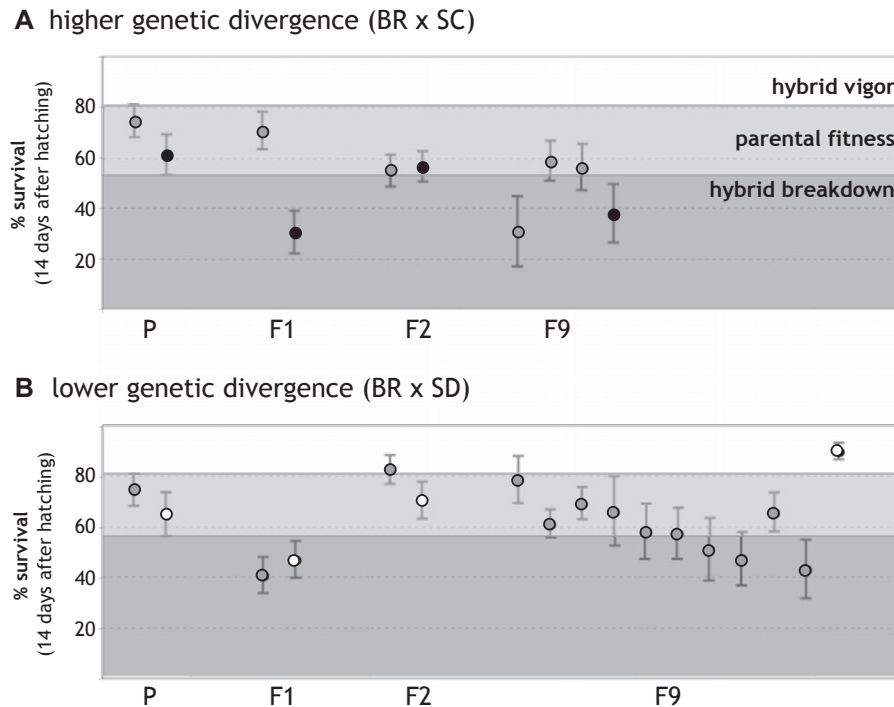


Figure 4. Intrinsic hybrid fitness at different levels of genetic divergence. The graphics refer to survivorship at 14 days (mean \pm 1 SE) in crosses between taxa with high (A) and low (B) genetic divergence. Each genotype is colored accordingly to its mitochondrion (SC: black; BR: gray; SD: white). “Parental fitness” is defined by the mean survivorship (\pm 1 SE) of parental taxa. Thus, higher survivorship is suggestive of “hybrid vigor” whereas lower survivorship is suggestive of “hybrid breakdown.”

reciprocal F1 and in three RILs with mean survivorship lower than parental fitness is not significant. All the exact *P*-values are listed in Table S4.

Finally, all parental inbred lines have higher mean fitness than the original parental populations but, with one exception, the increase of intrinsic fitness is not significant (Fig. S1).

IMPACT OF EXTRINSIC AND INTRINSIC SELECTION IN DIFFERENT RILS

We summarized intrinsic and extrinsic fitness for a subset of four and 10 lines, respectively for crosses between divergent and similar taxa (Fig. 5). As indicated by the previous results (Figs. 3, 4), hybrid lines resulting from ecologically and genetically divergent taxa (BR \times SC) have similar extrinsic fitness to their respective parentals, but lower intrinsic fitness. In contrast, hybrid lines resulting from ecologically and genetically similar taxa (BR \times SD) always have higher extrinsic fitness than respective parentals, but variable intrinsic fitness.

Discussion

HYBRIDIZATION LEADS TO TRANSGRESSIVE SEGREGATION IN HEAT TOLERANCE

Ecologically relevant traits often have a polygenic basis that can vary among closely related taxa. Thus, the same phenotype in

different parental taxa might rely on the fixation of alternative alleles at different loci rather than sharing the same molecular basis (e.g., Manceau et al. 2010). Transgressive segregation results from the recombination of these alleles at quantitative trait loci (QTL), so that when they sum up, an extreme trait value arises in hybrids—that is, complementary gene action (Rieseberg et al. 1999; Rieseberg and Willis 2007). Previous work in *T. californicus* (Willett 2012a) had shown that F1 hybrids from several interpopulation crosses frequently show increased heat tolerance relative to parentals, but this effect often disappears at F2 hybrids. Thus, until now there was no evidence that hybridization in *Tigriopus* could lead to transgressive segregation in heat tolerance.

In agreement with previous work (Willett 2012a), our results show an increase of heat tolerance in F1 hybrids in the two crosses tested, which is not always maintained in F2 generation (Fig. 3). This result is likely due to the heterosis effects, which is most pronounced in first-generation hybrids. To test for heritable complementary gene action in hybrids—or transgressive segregation—we focus on thermal-tolerance in F9 hybrid lines, which are each characterized by having a unique mosaic of the genetic variation of the two parental populations while having reduced heterozygosity due to five generations of full-sib matings. Our results do not show evidence for transgressive segregation in the cross between heat-tolerant and sensitive populations (Fig. 3).

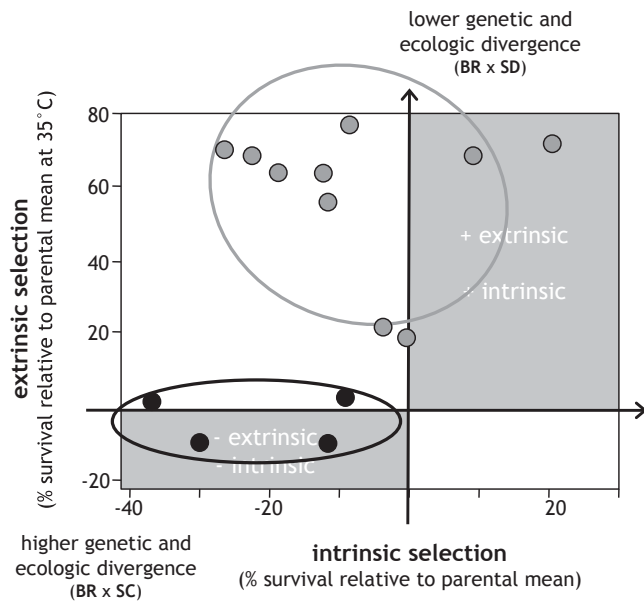


Figure 5. Extrinsic and intrinsic selection associated with recombinant genotypes. Each dot represents an inbred line resulting from hybridization between taxa with high ecologic and genetic divergence (black) or between taxa with low ecologic and genetic divergence (gray). Data for extrinsic and intrinsic fitness of each recombinant were standardized to the midparent means, so that lines with mean close to zero have equivalent fitness to the respective parental taxa. The circles define a density ellipse probability of 0.75.

In contrast, it is in crosses between the two heat-tolerant taxa that we find evidence for transgressive segregation. When parental taxa are at their sublethal temperature (Fig. 3B, 35°C), the majority of recombinant inbred lines outperform both parentals (9 of 12 lines). Moreover, we show that three of these recombinant genotypes are tolerant to temperatures that are lethal to both parentals (Fig. 3B, 36°C) and at which heterozygosity of F1s did not provide increased fitness. This evidence for transgressive segregation in an ecologically relevant trait such as tolerance to heat-stress suggests that these hybrid lines can potentially occupy fitness peaks on the local adaptive landscape that were previously underused by parental taxa.

Adaptation to laboratory conditions could potentially provide an alternative explanation for increased thermal tolerance in F9 RILs relative to the original populations. The most direct test to distinguish transgressive segregation from adaptation to the laboratory would require testing for increased heat tolerance in parental inbred lines (PILs). Such test was prevented by the low fecundity of the PILs, likely a reflection of inbreeding depression previously documented in natural populations of *T. californicus* (Brown 1991; Palmer and Edmands 2000). However, two lines of evidence suggest that such an increased thermal tolerance in hybrids results from transgressive segregation rather than adapta-

tion to laboratory conditions. First, Kelly et al. (2011) explicitly tested if artificial selection in the laboratory could increase thermal tolerance of eight populations of *T. californicus*. They found little response in heat tolerance even after 10 generations of strong selection and concluded that standing variation for thermal tolerance is low in natural *T. californicus* populations (Kelly et al. 2011). Given that direct selection failed to yield increased thermal tolerance, it is somewhat unlikely that thermal tolerance would spontaneously evolve as a pleiotropic consequence to unknown selective forces in the lab environment. Rather, we believe that the recombinant gene pool in the RILs provides novel genetic variation that resulted in novel thermal phenotypes. Second, if increased thermal tolerance evolved in response to generations of rearing in the laboratory environment, we would have expected both experimental crosses to yield similar results. Instead, our data show that increased heat tolerance only occurs in RILs between two tolerant populations ($P < 0.01$, Fisher's exact test). Together, these observations suggest that thermal tolerance results from hybridization and complementary gene action between thermal tolerant populations—that is, transgressive segregation.

The amount of transgression might be expected to increase either as a function of genetic distance between parental taxa, which leads to neutral fixation of QTLs in parental taxa with time since isolation, or as a function of phenotypic similarity, which leads to adaptive fixation of QTLs due to stabilizing selection (Rieseberg et al. 1999; Stelkens and Seehausen 2009). In our experimental crosses we only find transgression in heat tolerance in the cross between heat-tolerant populations, suggesting that phenotypic similarity, rather than genetic differentiation, might be facilitating transgression in *Tigriopus*. Extending our assays to multiple crosses between populations with various degrees of genetic and ecological divergence should allow us to distinguish between the two kinds of divergence, and to accurately test this hypothesis.

The adaptive potential of transgressive segregation in hybrids has long been recognized in agricultural breeding programs, but only more recently it became perceived as an important mechanism for species diversification (Seehausen 2004; Dittrich-Reed and Fitzpatrick 2013). Recent studies in experimental hybrids have demonstrated that transgression may occur in several ecologically relevant traits such as morphological traits (skull and body morphology in cichlid fish; Albertson and Kocher 2005; Stelkens et al. 2009), physiological traits (salt tolerance in *Helianthus* sunflowers; Lexer et al. 2003), life-history traits (flowering time in *Arabidopsis*; Clarke et al. 1995), and behavioral traits (mating in *Drosophila* and explorative strategies in house mouse; Ranganath and Aruna 2003). Moreover, studies in natural populations have shown that hybridization can result in the evolution of new niches and lead to the formation of hybrid species (*Helianthus* sunflowers and sculpins; Lexer et al. 2003; Nolte et al. 2005). Our results

in the copepod *T. californicus* corroborate these previous findings by showing that hybridization can also lead to transgressive segregation in heat tolerance, an important ecological factor limiting species ranges in nature.

CYTONUCLEAR INCOMPATIBILITIES CAN ARISE AT EARLY STAGES OF POPULATION DIVERGENCE

Cytonuclear incompatibilities constitute a form of Dobzhansky–Muller incompatibility (DMI) that can quickly arise during allopatric divergence due to the faster evolutionary rate of the mitochondrial DNA and to its intimate interaction with nuclear-encoded proteins. Recent work suggests that cytonuclear DMIs may be more common than previously recognized (Rand et al. 2004; Burton and Barreto 2012), and that they may evolve at early stages of divergence between genetically structured populations. In *T. californicus*, the mitochondrial DNA evolves notably faster relative to other organisms (Willett 2012b), and cytonuclear DMIs are known to occur in several interpopulation crosses. Yet, parental populations showing hybrid breakdown are typically characterized by very high levels of mitochondrial divergence (>17% of sequence divergence in COI and >19% in *cytb*; Edmands 1999, but see Burton 1990).

Our results (Fig. 4) show that the cross between the two populations at intermediate stages of divergence (BR × SD; 10.4% sequence divergence in *cytb*) shows some hybrid breakdown, but at lower magnitude than the cross between the two highly divergent populations (BR × SC; 20.2% sequence divergence in *cytb*). In the context of previous work involving multiple populations (Edmands 1999), our results suggest that cytonuclear DMIs can evolve very early during species formation in *T. californicus*. Yet, adding more crosses at intermediate levels of divergence such as the one used here is necessary to establish a generalization concerning the evolution of these cytonuclear incompatibilities.

In addition, our results with crosses involving the previously untested BR (Bird Rock) population show that F1-vigor followed by F2-breakdown is not always the case in *Tigriopus*, contrary to other interpopulation crosses (Edmands 1999; Ellison and Burton 2008). In both crosses, one or both reciprocal F1 hybrids are affected by hybrid breakdown, and fitness recovered to parental levels in the F2 generation (Fig. 4). Although this is unusual in *T. californicus*, this has been the rule in many other systems subjected to systematic hybridization experiments (e.g., in fruit flies, fishes, and mice; Coyne and Orr 1989; Bolnick et al. 2008; Good et al. 2008). We speculate that in our interpopulation crosses of *Tigriopus*, this pattern is due to a dominance of some BR alleles in comparison to other two populations, but further crosses involving BR and other populations are needed to test this hypothesis. Another important finding is that the hybrid breakdown reported here appears to be asymmetric. F1 hybrids with SC (Santa Cruz) mtDNA have significantly lower fitness

than its reciprocal carrying BR mtDNA ($P = 0.014$). Although this asymmetry is also unusual relative to most of the crosses previously reported in *Tigriopus* (but see Willett and Burton 2001; Ellison and Burton 2008), this constitutes a central theoretical expectation for DMIs involving uniparentally inherited genetic factors such as the mitochondria, and has been reported in various systems throughout the tree of life (also known as the “Darwin’s corollary”; Turelli and Moyle 2007).

Together, these results suggest that the cytonuclear incompatibilities can arise at early stages of divergence in *T. californicus*. Also, these results confirm the theoretical expectation that the stochastic differences in the accumulation of cytonuclear DMIs can result in asymmetric postzygotic barriers to gene flow (Turelli and Moyle 2007).

HYBRIDIZATION CAN PRODUCE GENOTYPES THAT ARE BENEFICIAL BOTH BY INTRINSIC AND EXTRINSIC SELECTION

The phenotypic variation generated by transgressive segregation can potentially enlarge the working surface for selection, and facilitate the evolution of novel adaptations where ecological opportunity exists. However, the potential benefit that hybridization provides on extrinsic fitness can only be evaluated in conjunction with its effect on intrinsic fitness. Previous studies in *Tigriopus* were limited in addressing this issue because they averaged fitness of F2 hybrids across different recombinants, and they did not integrate intrinsic and extrinsic selection in different genotypes. Here RILs were used to examine the genetic variation that arises during hybridization because (1) they provide mosaic genotypes of parental taxa while reducing the confounding effect of heterozygosity; and (2) they allow for replicated measurements of intrinsic and extrinsic fitness of genetic lineages with reduced interindividual variation.

Because there has been no extrinsic selection during the development of the RILs, they are expected to represent a random assembly of the parental genes that underlie heat tolerance (extrinsic selection); the same is not expected for genes underlying cytonuclear incompatibilities (intrinsic selection). During the three generations of outcrossing, genotypes with hybrid sterility or high inviability will decrease in frequency. In addition, during the following five generations of inbreeding, RILs carrying alleles causing outbreeding or inbreeding depression will tend to die off before reaching F9 generation. Thus, the RILs obtained represent a subset of the hybrid lines with higher intrinsic fitness. Our results with parental inbred lines confirm this expectation (Fig. S1). Despite this bias toward lines with higher fitness, in both crosses we were able to sample F9 lines with mean fitness below that of parentals, and some with the lowest values of fitness observed at F1 generation (Fig. 4). Thus, at least some of the sampled F9 RILs show the same level of hybrid breakdown that characterizes early

generational hybrids, whereas other RILs have recovered fitness to the level of parental taxa.

Our results show that, when we consider both extrinsic and intrinsic selective forces we observe that the outcome of selection varies strongly among RILs and between the two crosses (Fig. 5). Relatively to parental taxa, the RILs between the more genetically and ecologically divergent taxa (BR × SC) are not affected by extrinsic selection, but are negatively impacted by intrinsic selection, probably associated with persistence of some DMIs in F9 hybrids. In contrast, the RILs between the genetically and ecologically similar taxa (BR × SD) are usually favored by extrinsic selection, and only a fraction of those is still affected by intrinsic selection. Thus, our results show that, in the cross between the less genetically and ecologically divergent taxa, a few generations of recombination are enough to generate genotypes characterized by transgressive segregation in ecological relevant traits like heat tolerance, and without apparent hybrid breakdown.

Natural populations of *Tigriopus* typically inhabit a system of temporarily connected tidal pools, functioning as a metapopulation within a single-rock outcrop (Dybdahl 1994). Individual pools experience high fluctuations in temperature, sometimes reaching lethal temperatures (Kelly et al. 2011) that can potentially drive local extinction of subpopulations within a *Tigriopus* metapopulation. Thus, the ecological conditions that benefit the recombinant genotypes between our heat-tolerant populations might be frequently found in their natural habitat. Previous work indicated that heat-tolerant southern populations of *T. californicus* have limited potential for adaptation to climate change and that minor increases in temperature may lead populations to extinction (Kelly et al. 2011). Although the opportunity for hybridization is low due to restricted gene flow between populations, our results suggest that when migration occurs, hybridization can potentially release these populations from their adaptive limits and provide a mechanism to respond to environmental change, even in the face of hybrid breakdown in early generational hybrids. We note that our RILs characterized by increased heat tolerance and no hybrid breakdown were generated in a noncompetitive environment, where hybrids are isolated from parental genotypes. Long-term hybridization experiments with these populations could be used to test whether the transgressive segregation in heat tolerance accompanied by elimination of genetic incompatibilities might generate evolutionarily stable hybrid lineages.

Hybridization in *T. californicus* has been considered largely maladaptive because early generational hybrids generally have shown intrinsic hybrid breakdown and intermediate heat tolerance. The results presented here show that in some instances, a few generations of hybridization can give rise to genotypes that are favored both by intrinsic and extrinsic selection. Our interpopulation crosses between the two ecologically and genetically similar taxa show that hybridization may (1) lead to novel pheno-

types in ecologically relevant traits by transgressive segregation; and (2) some of the hybrid genotypes characterized by transgression are not affected by hybrid breakdown. These findings support the view that hybridization can provide a valuable source of evolutionary novelty, generating ecological traits that may be advantageous in the face of environmental change.

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LITERATURE CITED

- Abbott, R. J. 1992. Plant invasions, interspecific hybridization and the evolution of new plant taxa. *Trends Ecol. Evol.* 7:401–405.
- Albertson, R. C., and T. D. Kocher. 2005. Genetic architecture sets limits on transgressive segregation in hybrid cichlid fishes. *Evolution* 59:686–690.
- Anderson, E., and G. L. Stebbins. 1954. Hybridization as an evolutionary stimulus. *Evolution* 8:378–388.
- Arnold, M. L. 1997. *Natural hybridization and evolution*. Oxford Univ. Press, Oxford, NY.
- Barton, N. H., and G. M. Hewitt. 1985. Analysis of hybrid zones. *Ann. Rev. Ecol. Syst.* 16:113–149.
- Bolnick, D. I., M. Turelli, H. Lopez-Fernandez, P. C. Wainwright, and T. J. Near. 2008. Accelerated mitochondrial evolution and “Darwin’s corollary”: asymmetric viability of reciprocal F1 hybrids in Centrarchid fishes. *Genetics* 178:1037–1048.
- Brown, A. F. 1991. Outbreeding depression as a cost of dispersal in the harpacticoid copepod, *Tigriopus californicus*. *Biol. Bull.* 181:123–126.
- Buerkle, C., R. Morris, M. Asmussen, and L. Rieseberg. 2000. The likelihood of homoploid hybrid speciation. *Heredity* 84:441–451.
- Burton, R. S. 1985. Mating system of the intertidal copepod *Tigriopus californicus*. *Marine Biology* 86:247–252.
- . 1990. Hybrid breakdown in developmental time in the copepod *Tigriopus californicus*. *Evolution*: 1814–1822.
- Burton, R. S., and F. S. Barreto. 2012. A disproportionate role for mtDNA in Dobzhansky–Muller incompatibilities? *Mol. Ecol.* 21:4952–4957.
- Burton, R. S., C. K. Ellison, and J. S. Harrison. 2006. The sorry state of F2 hybrids: consequences of rapid mitochondrial DNA evolution in allopatric populations. *Am. Nat.* 168:S14–24.
- Clarke, J. H., R. Mithen, J. K. Brown, and C. Dean. 1995. QTL analysis of flowering time in *Arabidopsis thaliana*. *Mol. Gen. Genet.* 248:278–286.
- Cossé, A. A., M. G. Campbell, T. J. Glover, C. E. Linn Jr., J. L. Todd, T. C. Baker, and W. L. Roelofs. 1995. Pheromone behavioral responses in unusual male European corn borer hybrid progeny not correlated to electrophysiological phenotypes of their pheromone-specific antennal neurons. *Experientia* 51:809–816.
- Coyne, J. A., and H. A. Orr. 1989. Patterns of speciation in *Drosophila*. *Evolution* 43:362–381.
- deVicente, M. C., and S. D. Tanksley. 1993. QTL analysis of transgressive segregation in an interspecific tomato cross. *Genetics* 134:585–596.
- Dittrich-Reed, D. R., and B. M. Fitzpatrick. 2013. Transgressive hybrids as hopeful monsters. *Evol. Biol.* 40:310–315.
- Dybdahl, M. F. 1994. Extinction, recolonization, and the genetic structure of tidepool copepod populations. *Evol. Ecol.* 8:113–124.

- Edmands, S. 1999. Heterosis and outbreeding depression in interpopulation crosses spanning a wide range of divergence. *Evolution* 53:1757–1768.
- . 2001. Phylogeography of the intertidal copepod *Tigriopus californicus* reveals substantially reduced population differentiation at northern latitudes. *Mol. Ecol.* 10:1743–1750.
- Edmands, S., and R. Burton. 1999. Cytochrome C oxidase activity in interpopulation hybrids of a marine copepod: a test for nuclear-nuclear or nuclear-cytoplasmic coadaptation. *Evolution* 53:1972–1978.
- Ellison, C. K., and R. S. Burton. 2008. Interpopulation hybrid breakdown maps to the mitochondrial genome. *Evolution* 62:631–638.
- Good, J. M., M. A. Handel, and M. W. Nachman. 2008. Asymmetry and polymorphism of hybrid male sterility during the early stages of speciation in house mice. *Evolution* 62:50–65.
- Grant, P. R., and B. R. Grant. 2011. How and why species multiply. Princeton Univ. Press, Princeton, NJ.
- Grant, V. 1981. Plant speciation. Columbia Univ. Press, New York.
- Harrison, R. G. 1993. Hybrids and hybrid zones: historical perspective. Pp. 3–10 in R. G. Harrison, ed. *Hybrid zones and the evolutionary process*. Oxford Univ. Press, New York.
- . 2012. The language of speciation. *Evolution* 66:3643–3657.
- Hedrick, P. W. 2011. *Genetics of populations*. Jones & Bartlett Publishers, Sudbury, MA.
- Kelly, M. W., E. Sanford, and R. K. Grosberg. 2011. Limited potential for adaptation to climate change in a broadly distributed marine crustacean. *Proc. R. Soc. Lond. Ser. B* 279:349–356.
- Lewontin, R. C., and L. C. Birch. 1966. Hybridization as a source of variation for adaptation to new environments. *Evolution* 20:315–336.
- Lexer, C., M. E. Welch, J. L. Durphy, and L. H. Rieseberg. 2003. Natural selection for salt tolerance quantitative trait loci (QTLs) in wild sunflower hybrids: implications for the origin of *Helianthus paradoxus*, a diploid hybrid species. *Mol. Ecol.* 12:1225–1235.
- Liou, L. W., and T. Price. 1994. Speciation by reinforcement of premating isolation. *Evolution* 48:1451–1459.
- Mallet, J. 2008a. Hybridization, ecological races and the nature of species: empirical evidence for the ease of speciation. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* 363:2971–2986.
- . 2008b. Mayr's view of Darwin: was Darwin wrong about speciation? *Biol. J. Linn. Soc.* 95:3–16.
- Manceau, M., V. S. Domingues, C. R. Linnen, E. B. Rosenblum, and H. E. Hoekstra. 2010. Convergence in pigmentation at multiple levels: mutations, genes and function. *Philos. Trans. R. Soc. B* 365:2439–2450.
- Muller, H. J. 1942. Isolating mechanisms, evolution and temperature. *Biol. Sympos.* 6:71–125.
- Nolte, A. W., J. Freyhof, K. C. Stemshorn, and D. Tautz. 2005. An invasive lineage of sculpins, *Cottus* sp. (Pisces, Teleostei) in the Rhine with new habitat adaptations has originated from hybridization between old phylogeographic groups. *Proc. Biol. Sci.* 272:2379–2387.
- Orr, H. A. 1995. The population genetics of speciation: the evolution of hybrid incompatibilities. *Genetics* 139:1805–1813.
- Palmer, C. A., and S. Edmands. 2000. Mate choice in the face of both inbreeding and outbreeding depression in the intertidal copepod *Tigriopus californicus*. *Mar. Biol.* 136:693–698.
- Presgraves, D. C. 2010. The molecular evolutionary basis of species formation. *Nat. Rev. Genet.* 11:175–180.
- Rand, D. M., R. A. Haney, and A. J. Fry. 2004. Cytonuclear coevolution: the genomics of cooperation. *TREE* 19:645–653.
- Ranganath, H. A., and S. Aruna. 2003. Hybridization, transgressive segregation and evolution of new genetic systems in *Drosophila*. *J. Genet.* 82:163–177.
- Rawson, P. D., and R. S. Burton. 2002. Functional coadaptation between cytochrome c and cytochrome c oxidase within allopatric populations of a marine copepod. *Proc. Natl. Acad. Sci. USA* 99:12955–12958.
- Rieseberg, L. H. 1997. Hybrid origins of plant species. *Annu. Rev. Ecol. Syst.* 28:359–389.
- Rieseberg, L. H., N. C. Ellstrand, and M. Arnold. 1993. What can molecular and morphological markers tell us about plant hybridization? *Crit. Rev. Plant Sci.* 12:213–241.
- Rieseberg, L. H., and J. H. Willis. 2007. Plant speciation. *Science* 317:910–914.
- Rieseberg, L., M. Archer, and R. K. Wayne. 1999. Transgressive segregation, adaptation and speciation. *Heredity* 83:363–372.
- Schoville, S. D., F. S. Barreto, G. W. Moy, A. Wolff, and R. S. Burton. 2012. Investigating the molecular basis of local adaptation to thermal stress: population differences in gene expression across the transcriptome of the copepod *Tigriopus californicus*. *BMC Evol. Biol.* 12:170. doi:10.1186/1471-2148-12-170.
- Seehausen, O. 2004. Hybridization and adaptive radiation. *Trends Ecol. Evol.* 19:198–207.
- Slatkin, M., and R. Lande. 1994. Segregation variance after hybridization of isolated populations. *Genet. Res.* 64:51–56.
- Stebbins, G. L. 1959. The role of hybridization in evolution. *Proc. Am. Philos. Soc.* 103:231–251. JSTOR.
- Stelkens, R. B., C. Schmid, O. Selz, and O. Seehausen. 2009. Phenotypic novelty in experimental hybrids is predicted by the genetic distance between species of cichlid fish. *BMC Evol. Biol.* 9:283. doi:10.1186/1471-2148-9-283.
- Stelkens, R., and O. Seehausen. 2009. Genetic distance between species predicts novel trait expression in their hybrids. *Evolution* 63:884–897.
- Turelli, M., and L. C. Moyle. 2007. Asymmetric postmating isolation: Darwin's corollary to Haldane's rule. *Genetics* 176:1059–1088.
- Willett, C. S. 2010. Potential fitness trade-offs for thermal tolerance in the intertidal copepod *Tigriopus californicus*. *Evolution* 64:2521–2534.
- . 2012a. Hybrid breakdown weakens under thermal stress in population crosses of the copepod *Tigriopus californicus*. *J. Heredity* 103:103–114.
- . 2012b. Quantifying the elevation of mitochondrial DNA evolutionary substitution rates over nuclear rates in the intertidal copepod *Tigriopus californicus*. *J. Mol. Evol.* 74:310–318.
- Willett, C. S., and R. S. Burton. 2001. Viability of cytochrome c genotypes depends on cytoplasmic backgrounds in *Tigriopus californicus*. *Evolution* 55:1592–1599.
- . 2003. Environmental influences on epistatic interactions: viabilities of cytochrome c genotypes in interpopulation crosses. *Evolution* 57:2286–2292.
- Willett, C., and J. Ladner. 2009. Investigations of fine-scale phylogeography in *Tigriopus californicus* reveal historical patterns of population divergence. *BMC Evol. Biol.* 9:139. doi: 10.1186/1471-2148-9-139.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Intrinsic fitness at parental inbred lines (PILs).

Table S1. Sample size, mean, and standard error for survivorship after heat-stress (extrinsic fitness).

Table S2. Corrected *P*-values for all relevant pairwise comparisons testing hybrid vigor in heat tolerance (extrinsic fitness).

Table S3. Sample size, mean, and standard error for survivorship during development (intrinsic fitness).

Table S4. *P*-values for all relevant pairwise comparisons testing hybrid breakdown (intrinsic fitness).