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RESEARCH ARTICLE

Long-term experimental hybrid swarms between nearly incompatible *Tigriopus californicus* populations: persistent fitness problems and assimilation by the superior population

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Abstract For the intertidal copepod *Tigriopus californicus,* outbreeding depression for a variety of fitness measures is typically observed in early-generation interpopulation hybrids. We examined both controlled crosses and long-term, freely mating experimental hybrid swarms composed of individuals from Baja California (Mexico) populations Playa Altamira and Punta Morro. In controlled crosses, F1 and F2 hybrids showed large and significant declines in hatching numbers compared to parentals, while reciprocal backcrosses produced no offspring at all. For long-term studies, four treatment groups were initiated: 100%PA, 100%PM, 50%PA: 50%PM, and 80%PA: 20%PM. Replicates were surveyed at 3-month intervals for morphometric, census and fitness measures. The PA and 80PA:20PM treatments had initial fitness below the PM treatment, and went extinct within the first 12 months of the experiment. The 50:50 treatment had fitness below the PM parent at the 3- and 6-month time points, recovered to equivalent or superior fitness from months 9 to 18, and dropped again below PM at month 21. Limited genotyping of diagnostic microsatellites was consistent with PM alleles going nearly to fixation in hybrid replicates and male morphological data were concordant with a shift toward PM values. Results were strikingly different from a recent study of a different pair of populations showing extensive introgression and superior fitness in hybrid populations. This demonstrates how long-term consequences of population mixing depend on the relative fitness and level of compatibility between hybridizing populations.

Keywords Fitness · Hybrid breakdown · Interpopulation hybridization · Morphology · Multiple generation · Outbreeding depression

Introduction

Human activity is increasingly causing disturbance of natural habitats as well as the relocation of species and populations by both intentional and accidental means. Intentional mixing of populations is sometimes proposed to 'rescue' taxa suffering from inbreeding depression or genetic erosion (Tallmon et al. 2004). While some intentional hybridizations appear to have been successful (e.g. Pimm et al. 2006; Finger et al. 2011), they run the risk of producing offspring that are intrinsically unfit or poorly adapted to local conditions (Hatfield and Schluter 1999; Huff et al. 2011). Accidental mixing of populations or species can also occur through the spread of invasive species (Bleeker et al. 2007; Gilchrist and Lee 2007), potentially threatening the biodiversity of native species (Barilani et al. 2005; Halis and Morley 2005; Muhlfeld et al. 2009). Anthropogenic or natural causes may bring together formerly allopatric species (Allendorf et al. 2001), and the collapse of multispecies assemblages into hybrid swarms may result in extinction of one or more parental species (Rhymer and Simberloff 1996; Seehausen et al. 2008). This phenomenon has been frequently referred to as genetic swamping, where genes of one species increase in frequency from the initial hybridization event until the genetic integrity of the second species is compromised

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(Bleeker et al. 2007; Childs et al. 1996; Kothera et al. 2007; Hedrick 2010). A loss of biodiversity due to swamping may be a particular danger when the species of concern comes into contact with a more common congener (Ellstrand and Elam 1993; Levin et al. 1996).

There are many factors that can impact the fitness of hybridizing populations including genetic divergence, reproductive compatibility and the relative fitness of each contributing parental group. Due to the extensive labor involved and longer generation time of many model species, it can be difficult to perform multigenerational studies, so experimental work often focuses on the effects of hybridization on early-generation crosses (Edmands 2007). Consequently, hybrid fitness can be difficult to predict particularly because the mixing of gene pools, followed by generations of recombination, can result in the formation of both beneficial and detrimental gene interactions. In some cases, hybrids may exhibit an increase in fitness termed heterosis or hybrid vigor, but hybridization may also result in a decrease in fitness known as outbreeding depression. This might occur in F1 hybrids due to the disruption of local adaptation, underdominance or epistatic interactions. Fitness declines may also be delayed until the F2 or backcross generations, when deleterious heterozygote × homozygote or homozygote × homozygote interactions arise (Lynch 1991; Turelli and Orr 2000).

The mixing of two previously isolated populations or species may result in a hybrid swarm, in which overlapping hybrid generations coexist in the same population (e.g. Blum et al. 2010; Roberts et al. 2011). Over time many different outcomes are possible including lasting outbreeding depression, stabilization, assimilation, or the creation of superior recombinant genotypes. In a previous study using two moderately incompatible *Tigriopus californicus* populations (Hwang et al. 2011), fitness and morphology of hybrid swarms were monitored over many generations. Results showed extensive introgression between populations, with hybrid inferiority in early generations followed by hybrid superiority in later generations. In the present study we tested whether the same outcome occurs following interactions between a pair of populations with greater genetic divergence, higher incompatibility in early generation hybrids and greater fitness differences under lab conditions.

Tigriopus californicus is an excellent system for studying experimental hybridization because it has a short generation time (~23 days) and is easily reared in the lab. This species inhabits rocky intertidal outcrops extending from Alaska down to central Baja California, Mexico. Despite a seemingly high potential for dispersal, populations are genetically differentiated over short geographic distances, with mitochondrial DNA differences that range from 0.2 to 23% (Burton and Lee 1994; Edmands 2001). We used nearly incompatible *T. californicus* populations from Punta Morro (PM) and Playa Altamira (PA) in Baja California, Mexico. These two populations show approximately 21% mitochondrial cytochrome oxidase I divergence (Edmands 2001) and partial incompatibility in that only one reciprocal cross will produce viable, fertile offspring. It could be argued that there are grounds for calling these two populations sibling species (Ganz and Burton 1995). However, there is no evidence for prezygotic isolation between these populations, and viable F1 and F2 offspring are produced for one of the two reciprocal crosses (Ganz and Burton 1995). Many studies of *T. californicus* have shown that interpopulation crosses result in F1 heterosis while F2 hybrids show reduced fitness (e.g. Burton 1986, 1987, 1990a, b; Edmands 1999), but the population pair used in this study does not conform to this previous pattern. Instead, it exhibits severe fitness reductions in both F1 and F2 hybrids.

In this study, we assessed the outcome of hybridization for two different mixed-population starting ratios over the course of 21 months of free mating (a maximum of about 27 generations). Patterns of fitness, morphology and genetic composition were assayed at 3-month intervals. Parental populations differed greatly in their ability to thrive in the laboratory environment and the final outcome of hybridization was either extinction of the entire swarm or apparent swamping by the superior population.

Methods

Population sampling

Populations were sampled from two locations in Baja California, Mexico: Punta Morro (PM, 31°52′N, 116°40′W) and Playa Altamira (PA, 28°32′N, 114°5′W) in May 2003 (animals used for controlled crosses) and in June 2004 (animals used for long-term hybrid swarms). Samples were maintained as mass cultures in 400 ml beakers with filtered seawater (37 μm) containing finely ground Spirulina (0.2 mg/ml) and housed in a 20°C incubator with a 12 h light:12 h dark cycle.

Tigriopus biology

The reproductive biology of *Tigriopus* has been well-documented (Egloff 1966; Vittor 1971). Mating and reproduction occurs year round. Adult males use their antennae to clasp virgin females and mate guard them until the females are sexually mature. Males have multiple matings while females mate only once and store sperm to fertilize multiple broods of offspring (Burton 1985). Individual lifespan may be as long as 95 days and females may

produce up to 20 clutches of eggs. Clutch size varies from less than 10 nauplii (larvae) to over 100 (pers. obs.; Vittor 1971) and minimum generation time is approximately 23 days at 20°C (Burton 1987).

Controlled crosses

Cultures were maintained in the laboratory for 12 months before controlled crosses began. The controlled crosses followed methods similar to those used in Edmands (1999) and focused on quantifying reproductive compatibility in a pair of populations known to have qualitatively lower compatibility and greater asymmetry between reciprocal crosses (Ganz and Burton 1995) than any of the population pairs tested by Edmands (1999). All crosses were conducted using coarsely filtered sea water (37 μm) containing 0.2 g ground *Spirulina* per liter. Virgin females and sexually mature males were collected by pipetting clasped pairs onto a piece of filter paper, and using fine needle probes to tease males and females apart. Control crosses (PA × PA and PM × PM) and hybrid crosses (PA female × PM male and PM female × PA male) were established by uniting 5 virgin females and 5 mature males in a single Petri dish. A total of 30–50 pairs were established for each of the four crosses. These mating dishes were checked every 2–3 days. When females with egg sacs were observed, they were isolated and placed individually into new petri dishes. These dishes were then examined every 2–3 days. When the first clutch of larvae hatched, the mother was transferred to a new dish and the number of live larvae was counted by pipetting each larva into a new dish. These dishes were set aside and the number of survivors was counted 14 days later. Subsequent clutches of larvae were collected and all larvae for a particular cross type (e.g. PA × PA F1) were pooled into a 500 ml beaker. Beakers were monitored every 2–3 days for the appearance of clasped pairs. Pairs were dissected apart and males and females placed in mating dishes for either F2 crosses (F1 × F1) or backcrosses (F1 × parental). Again, dishes were monitored every 2–3 days and females with egg sacs were transferred to their own individual Petri. The number of larvae in the first clutch was counted and the number of survivors was recounted 14 days later. In this way, generations were made discrete.

Long-term hybrid swarms

Four different culture treatments were set up for this experiment: 100%PA, 100%PM, 50%PA:50%PM, and 80%PA:20%PM. We recognize that an optimally balanced design would have included a 20%PA:80%PM treatment, but the time-intensive nature of these experiments limited us to four treatments. Five replicates per treatment were each initiated by placing 500 gravid females in 1000 ml beakers containing 800 ml culture medium (400 ml *Platymonas* algal culture and 400 ml *Monochrysis* algal culture, supplemented with 0.16 g finely ground *Spirulina* and Tetramin flakes). All offspring were allowed to mate randomly for subsequent generations. Beakers were housed together in one incubator at 20°C set to a 12 h light:12 h dark cycle. Once every 2 weeks a 50% culture medium change was performed. At the same time beakers were also rotated within the incubator. Every 3 months a census estimate was taken for each replicate. This was performed by pouring the contents of an entire beaker into a 1 l plastic bottle. The bottle was gently inverted several times to evenly distribute copepods, after which 200 ml of culture was poured into a 600 ml transparent Gladware container. A light box was used for visual assistance in sorting copepods into males, females with eggs, pairs and subadult categories using a Pasteur pipet. Each category was counted and all individuals were returned to source beakers. Every 3 months, up to 20 gravid females and 20 mature males were removed from each replicate beaker and were used for morphometric assays. Females were also used for fitness assays. All copepods were returned to their source beakers after assays were completed. Replicates were maintained for up to 30 months. At two different time points, month 18 and month 30, 20 males and 20 females from surviving beakers were frozen for later molecular analyses.

Fitness assays

At each 3 month interval, 20 gravid females were sampled from each replicate and isolated into individual Petri dishes containing 11 ml filtered seawater 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 color. Each dish was monitored once daily until eggs hatched. On the day of hatching, 10 larvae per clutch were pipetted into a new dish with fresh seawater culture medium. Fourteen days later, individuals in each dish were counted to determine survivorship.

Morphological assays

Morphometric measurements were taken from digital images of adult copepods following procedures in Edmands and Harrison (2003). At each 3-month interval, up to 20 females and 20 males were randomly chosen from each replicate. All measurements were done at a magnification of ×32 using a Leica MZ12 dissecting microscope. Digital images were captured and morphological measurements were taken using Optimas 5.2. Absolute size was

calibrated using a stage micrometer. Eight measurements were taken for males: cephalothorax length (CTL), cephalothorax width (CTW), urosome length (UL), urosome width (UW), telson width (TW), caudal seta length (CSL), antennule width (AW) and clasper width (CLW). Four of the same measurements were taken for females (CTL, CTW, UW and AW). Egg sac length and area was also measured for each female. Every 3 months up to 40 individuals were scored from each replicate.

Microsatellite assays

Four microsatellite loci (Harrison et al. 2004) were found that exhibit alleles diagnostic for populations PA versus PM based on screening 40 individuals from each population. The four loci have been mapped to different chromosomes (Pritchard et al. 2011; Foley et al. 2011). Additional loci were sought but, since PCR primers were originally designed for highly divergent *T. californicus* populations, several loci could not be consistently amplified in the PA and/or PM populations. Diagnostic loci were screened in hybrid replicates at months 18 and 30. DNA was extracted from individual copepods using the lysis protocol described in Edmands et al. 2005. Individual copepods were incubated in 50 µl lysis buffer at 65°C for 1 h followed by 100°C for 15 min. Polymerase chain reactions were carried out in 12 µl volumes containing 0.5 µl template DNA, 0.25 µM fluorescently labeled forward primer, 1 µM reverse primer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 10 mM Tris–HCl, 50 mM KCl and 0.3 U *Taq* polymerase. Temperature cycling was as follows: 5 min denaturation at 94°C; 35 cycles of 30 s at 94°C, 35 s at 55°C, and 30 s at 72°C; 5 min at 72°C. Fluorescently labeled PCR products were run on a Beckman-Coulter CEQ 8000 Capillary Sequencer according to commercially recommended protocols. Allele sizes were compared to the manufacturer-produced 400 bp size standard and scored by eye.

Statistical analyses

Analyses of morphological and fitness characters within and between experimental population treatments were done using Statistica 7.1 (StatSoft, Tulsa, OK). Nested analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) were used to quantify differences in measures among the different treatment types. When appropriate, Bonferroni post hoc tests were utilized to determine the statistical significance between group means.

For each locus that appeared to be fixed for PM alleles, a binomial probability calculator (http://faculty.vassar.edu/lowry/binomialX.html) was used to determine the power of detecting PA alleles (1-β) if they were present at frequencies observed in other replicates or at other loci.

Results

Controlled crosses

Fitness (hatching and survivorship)

For control crosses, the PA replicates had a slightly lower clutch size and much lower survivorship than the PM controls. No offspring were produced in one of the two reciprocal crosses (PA female × PM male) and very few offspring were produced in the other reciprocal cross (PM female × PA male). Proportional clutch size in the successful cross (Fig. 1a) showed large (77–99%) and significant ($P < 0.0001$ for both, planned linear contrasts) declines from both parental groups in the F1 and F2 cohorts. Both reciprocal backcross cohorts produced no offspring. Of the individuals that did hatch (three clutches out of 63),

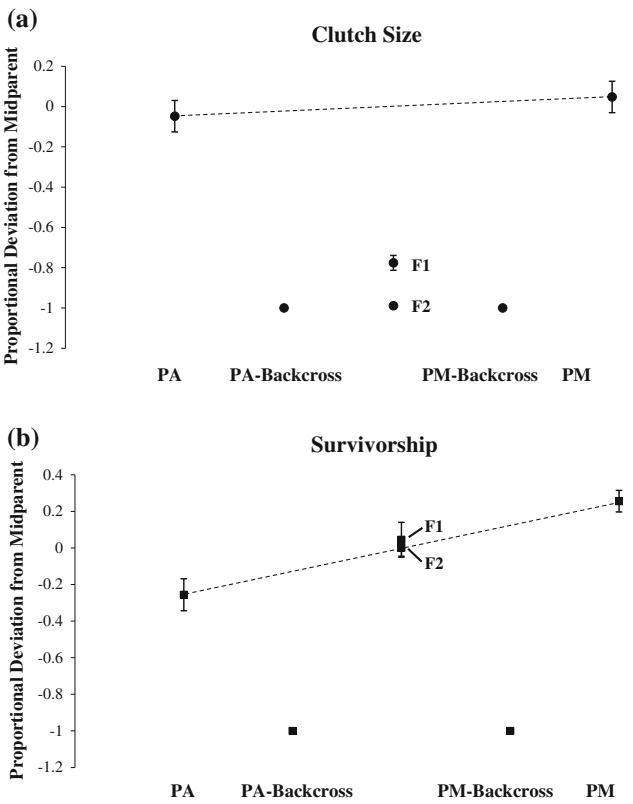

Fig. 1 Proportional deviation from midparent values [(replicate − midparent)/midparent] for clutch size (**a**) and survivorship (**b**) in two-generation controlled crosses. Values are averages among individual clutches relative to the midparent assayed in the same generation, ±1 SE. Hybrid individuals are offspring of PM females crossed with PA males, as the reciprocal cross produces no offspring. The *dashed line* indicates the additive expectation for each cohort

outbreeding depression was not observed in the survivorship counts of the F1 and F2 cohorts (Fig. 1b). Planned linear contrasts showed that differences between F1 and midparent as well as F2 and midparent survivorship were not significant ($P = 0.670$ and $P = 0.962$).

Long-term hybrid swarms

Census

Census counts show the low success and eventual loss of all PA and 80PA:20PM replicates (Fig. 2). Census averages included dead replicates with a count of zero. By month 12, all PA parental replicates as well as all 80PA:20PM replicates had gone extinct. While census counts for individual replicates showed large fluctuations over time (Fig. 2), there was a significant correlation between consecutive time points ($r = 0.34$, $P = 0.006$) indicating some potential for future census size to be predicted from current census size. Because PA replicates died out, the 50:50 and 80PA:20PM hybrid swarm treatments were compared to the surviving PM mean. Census counts from both hybrid treatments were lower than the PM mean at month 3. At months 6–18 at least two surviving 50:50 replicates had census counts greater than the PM mean. At month 18 two 50:50 replicates exceed the PM mean by at least 4 fold but, at month 21, the mean of the 50:50 replicates fell below the PM parent.

Survivorship

For each time point, survivorship was compared among treatments by nested ANOVA with Bonferroni post hoc corrections (Table 1). Both the PA and 80:20 treatments were significantly below the PM treatment at the 3- and 6-month time points and subsequently died out. The 50:50 treatment was also significantly below the PM treatment at 3 and 6 months but then recovered to values equivalent or superior to PM from months 9–18 before dropping to significantly lower survivorship at the final 21-month time point (Table 1; Fig. 3).

Survivorship of individual hybrid replicates was also compared to the superior parent. A one-way ANOVA was performed with all parental replicates of the same treatment grouped together. Bonferroni post hoc tests were used to determine whether hybrid replicates differed in survivorship from parental treatments. In all significant comparisons, hybrid replicates had lower survivorship than the superior parent. For the 50:50 treatment, however, more replicates showed equivalent survivorship to the superior parent (PM). All mixed population replicates proceeded through the first 3–6 months with survivorship equal or lower to the PM parent. After month 6, 80PA:20PM replicates crashed and survivorship could not be assayed. From month 9 to 18 all 50:50 replicates were equivalent to PM for survivorship. This was followed by one 50:50 replicate with lower survivorship ($P = 0.015$) at month 21.

Morphological assays

Nested ANOVA was performed at each time point to assess morphological variation, with treatment means compared to the superior parent, PM (Figs. 4, 5; Supplementary Tables 1, 2). Both male and female morphometric characters showed erratic temporal patterns of deviation from the superior parent in the first 12 months of the study. In males, the 50:50 treatment tended to parallel the PM treatment for the final 3 time points. Females in the PM and 50:50 treatments generally became smaller over time. At the final time point (21 months), both the PA and 80:20 treatments had died out, and the 50:50 treatment had become smaller for one character (UL) and larger for two characters (ESA and ESL). The opposite pattern was found in males with an overall trend for increased measures, with the exception of AW and UW. For the 50:50 treatment, these increases were significantly different between months 3 and 21 for five out of six characters (everything but telson width). CLW decreased significantly in

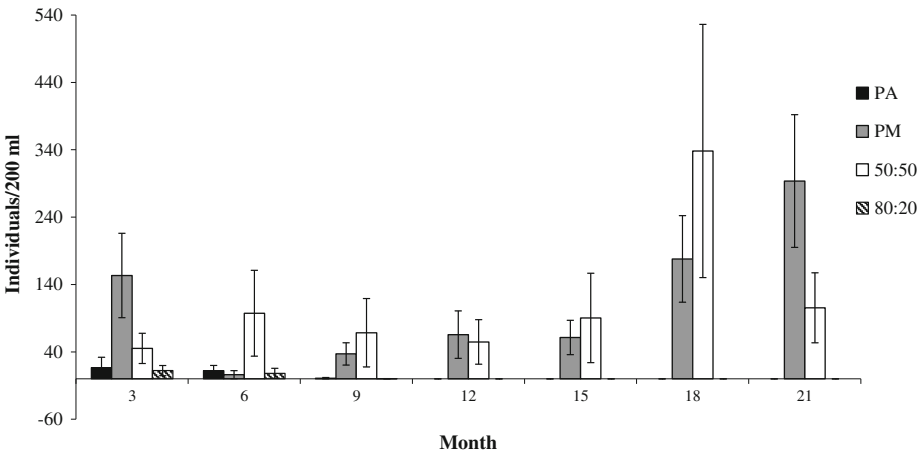

Fig. 2 Mean census counts among all five replicates for each treatment over 21 months of free mating. One replicate from PA population remained at month nine but died out before month 12, while 80PA:20PM replicates did not survive past month 6. *Error bars* represent 1 SE

Table 1 Survivorship comparisons among treatments by nested ANOVA and Bonferroni post hoc at each time point

Month	PM—PA	PA—50:50	PA—80:20	PM—50:50	PM—80:20	50:50–80:20
3	**0.4260**	−0.1857	−0.0769	**0.2403**	**0.3491**	0.1088
6	**0.2353**	−0.0169	0.1446	**0.2184**	**0.3799**	0.1615
9	−0.1594	−0.0177	–	−0.1771	–	–
12	–	–	–	−0.0022	–	–
15	–	–	–	**−0.0827**	–	–
18	–	–	–	0.0225	–	–
21	–	–	–	**0.2525**	–	–

For each month, the difference between treatment means is shown. Significant P values ($\alpha = 0.05$/number of tests performed for that month = 0.008 for months 3 and 6, 0.017 for month 9 and 0.05 for months 12–21) are indicated in bold. Dashes indicate comparisons that were not done because one or both treatments died out

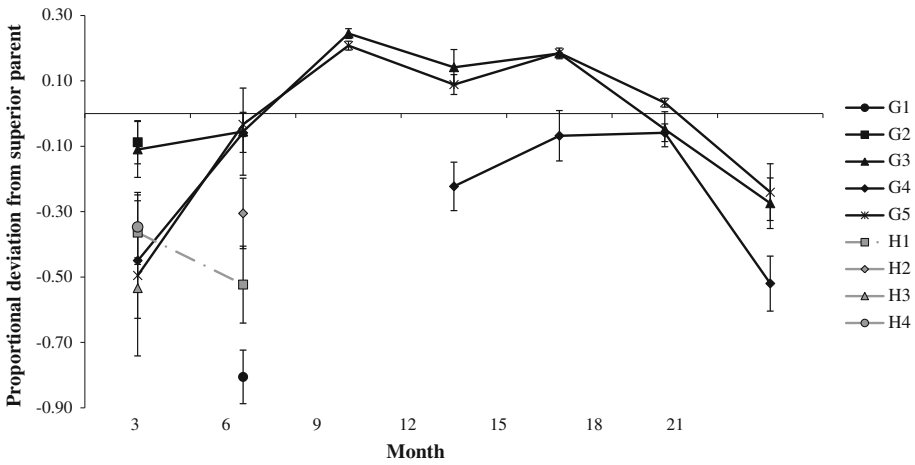

Fig. 3 Mean proportional deviation [(replicate − superior parent)/superior parent] for survivorship of each hybrid swarm replicate over 21 months of free mating. Values are averages among individual clutches relative to the superior parent assayed in the same generation. *Error bars* show 1 SE

size over time in the PM treatment ($P < 0.001$) but showed no significant change in the 50:50. By month 21, when only the 50:50 and PM treatments remained, the only significant difference between the two was smaller AW.

Nested ANOVA was performed across all time points to assess morphological variation. A multivariate test indicated a significant effect of both treatment and replicate for females and males. Both month and treatment had a significant effect on all four morphological characters measured in females as well as the two egg sac measurements. Out of the eight male morphological characters, all eight showed a significant month effect and seven differed significantly among treatments. One-way MANOVAs were run for each treatment by month for all measurements (Table 2). Fourteen out of 18 possible female treatment groups returned a significant p-value for replicate effect (Wilks' test, $P < 0.05$) and 12 of those were highly significant ($P \leq 0.0001$). For male replicates 13 out of 17 different treatment groups had a significant replicate effect.

Microsatellite identification

At month 18 (Table 3), four loci were genotyped for a small (<20) number of individuals from each of 50:50 replicates 3 and 5. In replicate 3 only locus 197 showed the presence of PA alleles at a frequency of 0.2. All other alleles were from the PM population. In cases where no PA alleles were genotyped, sample sizes corresponded to a 94.5–97.2% chance of detecting at least one PA allele at a frequency of 0.2. At month 30 (Table 3), two loci were genotyped for 33 individuals from 50:50 replicate number 3. PA alleles were present only at locus 197 at a frequency of 0.03. Locus 197 was also genotyped in replicate 4 and PA alleles were present at a frequency of 0.04. No homozygotes for PA alleles were found in any observed replicate for both months 18 and 30.

Discussion

Do short-term controlled crosses predict long-term hybrid swarm results?

The PA population was found to be inferior to the PM population, both in the short-tern crosses and in the long-term hybrid swarms. It is not clear why the PA population could be maintained under laboratory conditions for 12 months before controlled crosses began, and yet all PA

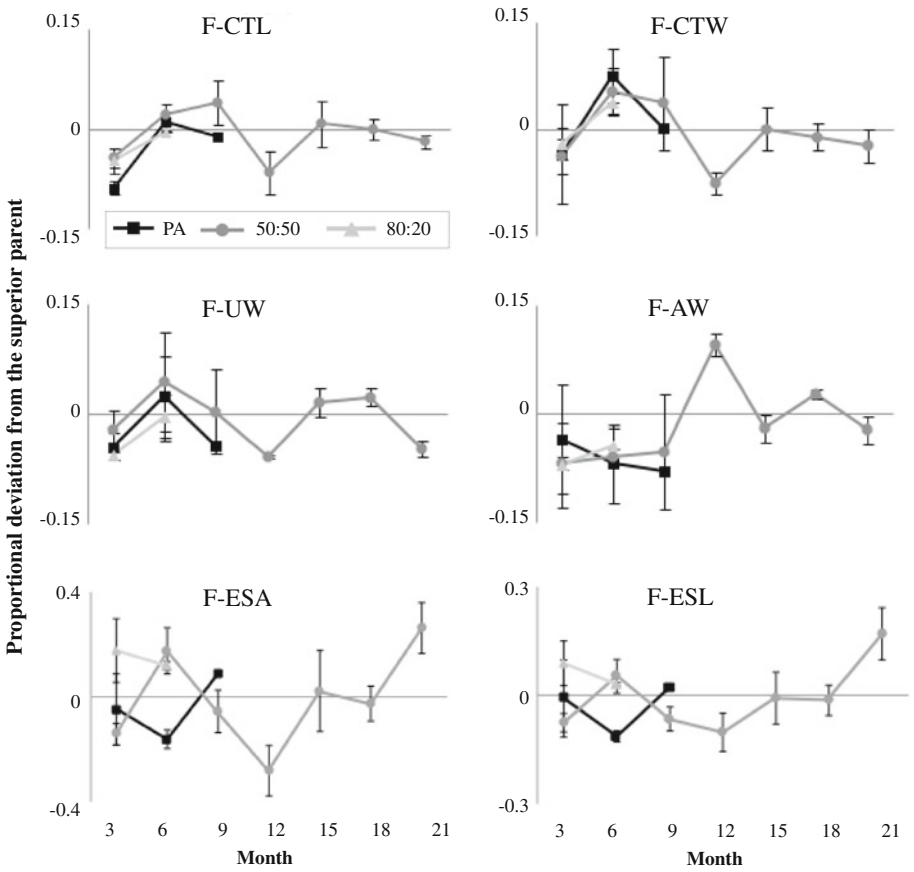

Fig. 4 Mean proportional deviation [(replicate − superior parent)/superior parent] for six morphometric characters in females from three treatments—PA, 50PA:50PM and 80PA:20PM. Standard errors among replicates are in parentheses with the exception of the PA treatment at month 9 where only one replicate remained

replicates died out within the first 12 months of the swarms experiment. It may be that the swarm replicates were more vulnerable due to smaller population sizes combined with the stress of biweekly water changes. It is also not clear why PA was inferior to PM in both short- and long-term experiments. One possibility is that the PM population is better-adapted to the relatively constant conditions of temperature and salinity maintained under laboratory culture. While PM was superior to PA in the small subset of fitness components measured (hatching number and 14-day survivorship in the short-term crosses, egg sac size and 14-day survivorship in the first 6 months of the long-term swarms) a more detailed analysis of life history components would be needed to better understand why the PA and PA-dominated swarm replicates went extinct.

Like Ganz and Burton (1995) we found hybrids produced in only one of the two reciprocal crosses between the PA and PM populations. In the successful cross, we found extremely low production of F1 and F2 hybrids, and no backcross hybrids. This low hybrid fitness, combined with relatively low fitness in one parental population, might forecast serious fitness problems in early hybrid swarm generations. Hybrid fitness problems might be expected to be aggravated by the apparent lack of prezygotic isolation between populations producing hybrids with low viability (Ganz and Burton 1995; Palmer and Edmands 2000). Indeed, the 80:20 treatment showed significantly depressed fitness for the first two time points, and all five replicates ultimately died out. In contrast, three of the five 50:50 replicates survived for the duration of the study, with one replicate showing depressed fitness at the first time point, apparent recovery of all surviving replicates at intermediate time points, and a return to depressed fitness for one replicate at the final time point. Given that controlled crosses showed extremely low hatching success in hybrids, as well as substantially higher survivorship in PM than in PA, one might expect the hybrid swarm replicates to go to complete fixation for PM alleles. Instead, molecular assays showed the persistence of PA alleles at low frequencies, even after 30 months of hybridization. The limited molecular data make it impossible to distinguish the extent to which PA individuals and/or alleles succeeded in the early generations of the experiment. However the persistence of these PA alleles, which likely have both beneficial and detrimental effects in different hybrid gene combinations (e.g. Edmands 2008; Edmands et al. 2009) may explain the significant fitness decline for one hybrid replicate in the final time point.

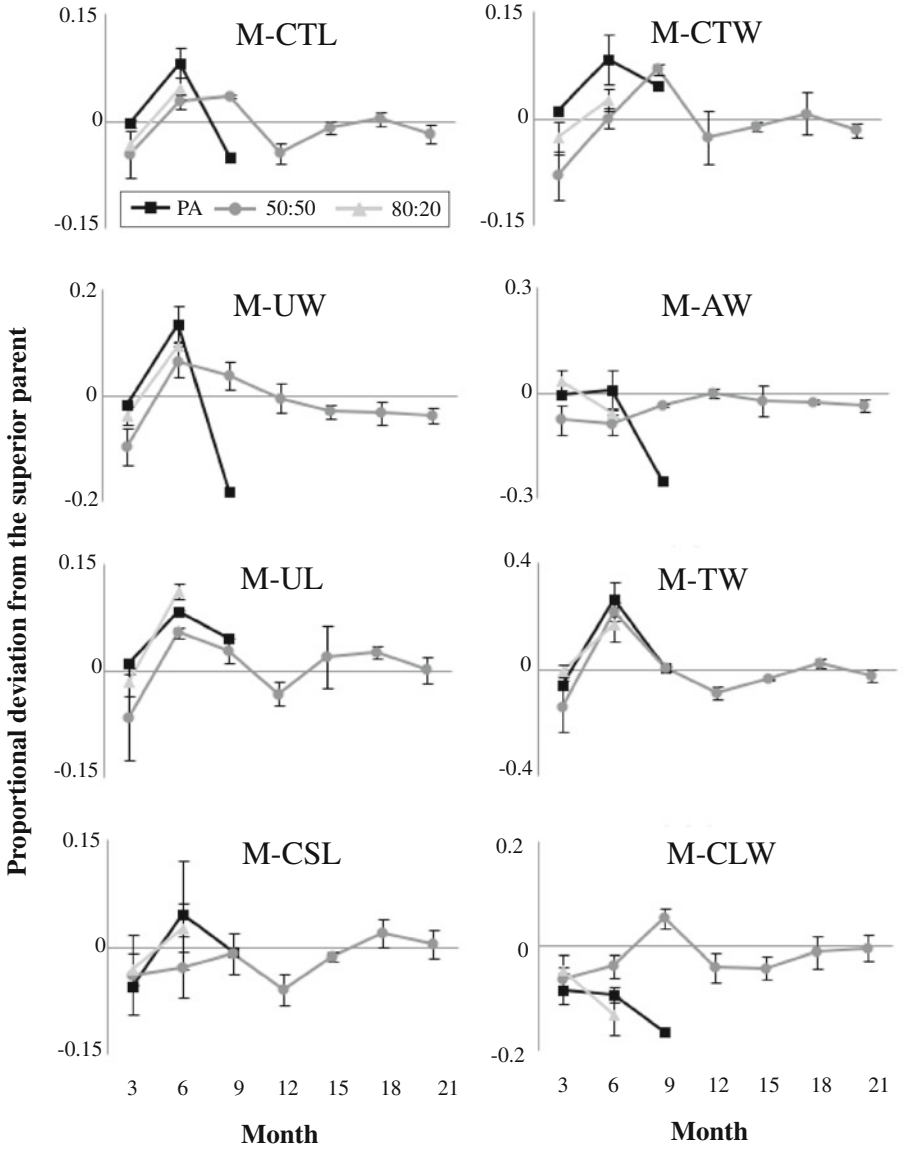

Fig. 5 Mean proportional deviation [(replicate − superior parent)/superior parent] for eight morphometric characters in males from three treatments—PA, 50PA:50PM and 80PA:20PM. Standard errors among replicates are in *parentheses* with the exception of the PA treatment at month 9 where only one replicate remained

Results of short-term controlled crosses help explain why long-term consequences of hybridization in the current study are very different from those in a previous study (Hwang et al. 2011). Compared to the current study, the two populations used in the previous study (RP and SD) had more similar fitness under laboratory conditions, and their F2 hybrids exhibited more moderate fitness reductions while their backcross hybrids exhibited no fitness reduction at all. This resulted in hybrid swarm populations showing extensive introgression and mild fitness reductions in early time points, followed by fitness levels that surpassed parentals in later time points. The substantial difference in the fitness of the first backcross generation may be particularly important in explaining the different outcomes of these two hybrid swarm studies, as it is homozygote-heterozygote interactions that are most likely to determine the fitness of later generation hybrids.

Is the outcome of hybridization determined by the level of divergence between hybridizing taxa?

In *T. californicus* there is no evidence of prezygotic isolation even at the highest levels of divergence (Ganz and Burton 1995; Palmer and Edmands 2000), while F2 hybrid breakdown increases in rough proportion to population divergence (Edmands 1999). This relationship is by no means perfect as the cross in the current study (PA × PMO, 21% COI divergence) showed much greater hybrid fitness problems (no live offspring in one reciprocal cross, 99% below the midparent in the F2 of the other

Table 2 Results of multivariate Wilks' test

Month	Females				Month	Males			
	F	Effect df	Error df	*P*		*F*	Effect df	Error df	*P*
3					3				
PA	1.838	6	11.00	0.1807	PA	One replicate measured			
PM	3.067	24	245.41	**0.0000**	PM	1.663	32	322.44	**0.0159**
50:50	2.481	24	168.66	**0.0004**	50:50	3.021	24	145.62	**0.0000**
80:20	6.328	12	94.00	**0.0000**	80:20	1.462	16	52.00	0.1506
6					6				
PA	2.455	6	25.00	0.0527	PA	3.006	8	14.00	**0.0345**
PM	0.805	24	70.98	0.7181	PM	0.608	8	11.00	0.7549
50:50	6.512	18	144.74	**0.0000**	50:50	1.794	24	116.61	**0.0217**
80:20	1.410	6	21.00	0.2572	80:20	1.428	8	20.00	0.2452
9					9				
PA	One replicate measured				PA	One replicate measured			
PM	3.473	24	168.66	**0.0000**	PM	2.212	16	94.00	**0.0095**
50:50	18.676	6	31.00	**0.0000**	50:50	1.449	8	31.00	0.2160
12					12				
PM	14.631	18	147.56	**0.0000**	PM	1.634	32	200.74	**0.0231**
50:50	7.870	12	102.00	**0.0000**	50:50	2.566	16	70.00	**0.0036**
15					15				
PM	5.227	24	287.27	**0.0000**	PM	2.430	32	233.93	**0.0001**
50:50	10.966	12	88.00	**0.0000**	50:50	2.679	16	98.00	**0.0015**
18					18				
PM	3.769	24	315.18	**0.0000**	PM	4.133	32	304.00	**0.0000**
50:50	1.974	12	104.00	**0.0339**	50:50	3.663	16	98.00	**0.0000**
21					21				
PM	3.634	24	203.55	**0.0000**	PM	3.743	32	300.31	**0.0000**
50:50	3.908	12	104.00	**0.0001**	50:50	1.949	18	96.00	**0.0203**

One-way MANOVA was performed for each treatment by month for all measurements. Significant tests are indicated in bold

Table 3 Microsatellite allele frequencies for 50:50 replicates obtained at months 18 and 30

Month	Replicate	Locus	*n*	Pop (allele)	Frequency	1-β
18	3	1,555	16	PM (168)	1	97.2
		197	10	PM (190)	0.8	89.3
				PA (202)	0.2	
		1,203	13	PM (202)	1	94.5
	5	197	16	PM (190)	1	97.2
		1,203	14	PM (202)	1	95.6
		1,202	16	PM (181)	1	97.1
30	3	197	32	PM (190)	0.97	62.3
				PA (202)	0.03	
		1,202	33	PM (202)	1	63.4
	4	197	34	PM (190)	0.96	64.5
				PA (202)	0.04	

1-β is the percent chance that a PA allele would be detected at least once given a frequency of 0.2 at 18 months and 0.03 at 30 months

reciprocal cross) than the most divergent cross assessed in previous studies (NB × SD, 22% COI divergence, F2 hatching success <40% below the midparent; Edmands 1999). Similarly, the long-term success of population mixing in the current study was substantially lower than that in a previous study of two populations that are only

mildly less divergent (RP × SD, 18% COI divergence; Hwang et al. 2011).

Beyond *Tigriopus*, other taxa often show a very rough correlation between population divergence and both pre- and post-zygotic isolation (reviewed in Edmands 2002). However, the rate by which incompatibilities accumulate seems to vary wildly across different taxonomic groups. For example, the evolution of hybrid inviability may take as little as 1.5–3.5 MY in some groups (Drosophila, anurans, sea stars, sea urchins and shrimp) and as much as 55 MY in groups such as birds (reviewed in Stelkens et al. 2009). It is difficult to place *Tigriopus* on this same time scale due to the absence of a fossil record. However, postzygotic isolation does appear to evolve slowly in *Tigriopus*, with viable and fertile hybrids produced in crosses up to a Nei's genetic distance of 0.842 (Ganz and Burton 1995), while in *Drosophila* the cutoff is reached at a Nei's genetic distance of 0.530 (Coyne and Orr 1997). One reason for the slow accumulation of incompatibilities in *Tigriopus* may be the absence of heteromorphic sex chromosomes (Ar-Rushdi 1963). Because the genes driving hybrid sterility and inviability are often X-linked (e.g. Coyne and Orr 1989) genic incompatibilities may accumulate more slowly in taxa lacking differentiated sex chromosomes (Rieseberg 2001).

Is the outcome of hybridization determined by the relative frequency of hybridizing taxa?

Several cases have been reported where rarer species are considered threatened by hybridization with a more common species (Childs et al. 1996; Levin et al. 1996; Rhymer and Simberloff 1996; Bleeker et al. 2007). Our *Tigriopus* results show that the outcome of hybridization is determined not just by initial population frequencies, but by the relative fitness of parents and their hybrids. In the present study an 80:20 mix of inferior: superior populations resulted in extinction, due either to hybrid fitness problems or the abundance of inferior parentals. In contrast, a 50:50 population mix resulted in apparent assimilation by the superior population (based on limited molecular data), with 60% of replicates surviving to the final 21-month time point. In our previous hybrid swarm experiment using populations with similar fitness levels (Hwang et al. 2011), there was no evidence of genetic swamping in either 50:50 or 80:20 replicates. In a different study of these same two populations which began with backcross hybrids (a 75:25 ratio), alleles from the rarer population increased in frequency in all four replicates (Edmands et al. 2005). Together these studies do not show a strong minority disadvantage to populations with initial frequencies as low as 20–25%, as long as the minority population is not competitively inferior.

Long-term success of freely mating hybrid swarms

If we view our experimental swarms as models for hybridization events of real conservation concern, their 'success' depends on the desired conservation goals. In our previous study of RP–SD swarms, surviving hybrid replicates were successful in achieving higher fitness than parentals, albeit at the cost of losing the genetic integrity of both original populations. In the present study, success of PA–PM swarms is less clear. If PM individuals were being added to an ailing PA population to rescue fitness, our results indicate that this strategy would be wholly unsuccessful at an 80PA:20PM ration, as all of our replicates went extinct. In the 50:50 treatment fitness was sufficiently improved to allow 60% of replicates to survive to the final time point, with the majority of surviving replicates showing fitness equivalent to the superior parent. However this partial fitness rescue came at the cost of apparent assimilation of the less fit population. The risk of genetic swamping may be particularly great in cases such as this where hybridizing populations are both highly incompatible and have differential fitness.

Wild populations versus observed patterns of morphology and fitness in experimental populations

Wild hybridization is often detected by the presence of morphological intermediates (Gompert et al. 2006; Norrmann 2009; Ureta et al. 2008). However, if swamping occurs, it becomes more difficult to detect an ancestral hybridization event with subsequent generations because individuals with hybrid ancestry may become indistinguishable in morphology from the superior parent. Four female morphological characters differed significantly between the parental populations at month 3, but only two of those were significantly different at month 6 and the PA parent did not survive beyond that point. This makes it difficult to assess whether or not morphological intermediates persisted throughout the experiment. Morphological measurements for females decreased over time. At month 21 50:50 replicates had larger egg sacs compared to the PM parent, as well as lower survivorship. This contrasts with our previous study on a different pair of populations in which females in long-term mixed populations showed higher survivorship but had smaller egg sacs (Hwang et al. 2011). Males also showed different morphological changes in the two studies. In the earlier study (Hwang et al. 2011) males showed an overall trend of decrease across treatments (with the exception of male caudal setae length) while in the current study male measurements either increased or remained the same over 21 months.

Overall, the morphometric signal for both males and females suggests that 50:50 morphology became more like

that of the PM parental over time, with this pattern being stronger in males. Molecular data are concordant with the overall shift toward PM genotypes over time, but also show that, even after 30 months, there is still a signature of the original hybridization event. Even though mixed populations look like the PM parent population and have similar fitness values, genetic analyses could still detect the presence of PA alleles in the population. This result supports the value of genotypic data in making management decisions to preserve the genetic integrity of individual populations or species. Even in the event of genetic swamping, it may be possible to determine which populations are descended from hybrids, provided that candidate parental populations can be identified.

Is the outcome of hybridization repeatable?

For managers attempting to restore multiple populations of the same species, it would be particularly useful to know how often similar hybridization events might result in the same outcome and how those patterns might differ among taxa. The experimental trials performed here provide an animal alternative to the known examples of plants that show repeated hybridization events leading to similar outcomes (Brochmann et al. 2000; Schwarzbach and Rieseberg 2002). These cases may involve specific characteristics such as chromosomal rearrangements, observed in hybrids of the genus *Helianthus*, that resist recombination in the parental species and thus contribute to observed repeatability between both synthetic lineages (Rieseberg et al. 1996) and natural and synthetic hybrids (Rieseberg et al. 2003). The overall implication from these studies is that strong deterministic forces are involved in driving the consequences of hybridization. Studies of *Tigriopus* have previously found only partial concordance among replicates of the same treatments for both molecular (Edmands et al. 2005) and morphological (Hwang et al. 2011) measures. This low repeatability may be due to the overriding effects of drift, as hybrid swarm replicates in a similar study using different source populations had effective population sizes ranging from 17 to 63 (Hwang 2009). In *Helianthus*, drift may also play an important role in the production of synthetic hybrids, which result from controlled crosses initiated with only a few individuals (Rieseberg et al. 1996). Drift probably plays a much smaller role in the creation of natural *Helianthus* hybrids, as wild populations of *Helianthus* species are estimated to have current effective population sizes on the order of millions (Strasburg and Rieseberg 2008). Compared to Tigriopus, the highly repeatable outcome of hybridization in *Helianthus* may therefore result from more efficient selection in natural populations and stronger selection on chromosomal rearrangements in both natural and synthetic hybrids.

Individual morphometric characters of each replicate in this study indicate that the path of morphological evolution is not necessarily repeatable. When morphological measurements are assessed together, replicates have distinct trajectories. Significant replicate effects were seen within treatments for all treatments after month 9. Replicate effects were significant for the 50:50 treatment for all months. Significant replicate effects were also observed for morphometric measures and survivorship for all months except month 18, indicating that the mean of all replicates for a treatment type may not be indicative of any particular replicate.

Two of the three 50:50 replicates had surprisingly parallel survivorship measures throughout the duration of the experiment. The individual replicates that did not go extinct were those with the fitness values greater than the superior parental controls at month 15. Further studies that integrate detailed accounts of both molecular and phenotypic data are warranted in order to resolve questions surrounding determinism and repeatability. Overall the results from this experiment support the notion that deterministic forces for morphological and molecular evolution may exist but be hampered by drift in the small populations used in this experiment, just as drift may impact natural populations experiencing extreme environmental fluctuations. A strong influence of drift relative to selection would be consistent with the limited evidence for local adaptation (Edmands and Deimler 2004; Hwang et al. unpublished data; but see also Willett 2010), the occurrence of maladapted gene complexes (Edmands et al. 2009) as well as levels of molecular subdivision that exceed measures of quantitative trait subdivision (Edmands and Harrison 2003).

Conclusions

Long-term consequences of hybridization are very difficult to predict. Our results suggest that information on the fitness effects of two generations of hybridization in a controlled cross can partially forecast the outcome of hybridization over many generations of free mating. Our previous work showed that mixing of moderately incompatible populations with similar fitness levels resulted in extensive introgression and the rise of superior hybrid genotypes in surviving replicates. Our present study showed that mixing of highly incompatible populations with different fitness levels resulted in extinction of replicates which started with a higher frequency of individuals from the less fit populations, and genetic swamping with partial fitness recovery in replicates which started with equal frequencies of the two populations. Overall results show that hybrid swarm populations can persist and sometimes thrive long after the effects of early-generation

outbreeding depression become evident (e.g. Templeton 1986; Rieseberg et al. 1996; Carney et al. 2000; Christiansen 2008). On a more cautionary note, hybrid swarm populations which appear to have recovered may subsequently decline, perhaps due to the persistence of alleles segregating on different hybrid backgrounds. Managers considering the intentional introduction of divergent individuals to bolster a dwindling population would ideally consider not only the level of divergence between hybridizing populations, but also the level of reproductive compatibility and the relative fitness of the populations in the intended environment.

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