Hybridization affects life-history traits and host specificity in *Diorhabda* spp.

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Abstract

Hybridization is an influential evolutionary process that has been viewed alternatively as an evolutionary dead-end or as an important creative evolutionary force. In colonizing species, such as introduced biological control agents, hybridization can negate the effects of bottlenecks and genetic drift through increasing genetic variation. Such changes could be beneficial to a biological control program by increasing the chances of establishment success. However, hybridization can also lead to the emergence of transgressive phenotypes that could alter host specificity; an important consideration when assessing potential non-target impacts of planned agents. In a series of lab experiments, we investigated the effects of hybridization between three species of *Diorhabda* released to control invasive *Tamarix* (saltcedar) on life history traits through two generations, and through the third generation for one cross. Depending on the cross, hybridization had either a positive or neutral impact on development time, adult mass, and fecundity. We evaluated preference for the target (saltcedar) relative to a non-target host *Tamarix aphylla* (athel), and found host specificity patterns varied in two of the three hybrids, demonstrating the possibility for hybridization to alter host preference. Importantly, the overall effects of hybridization were inconsistent by cross, leading to unpredictability in the outcome of using hybrids in biological control.

Keywords:
Biological control
Life-history traits
Host specificity
Hybrid vigor
Inbreeding
Invasive species

1. Introduction

Hybridization is an influential evolutionary process that has been viewed alternatively as an evolutionary dead-end, because hybrids are often less fit than the parental species (Mayr 1963; Dobzhanski 1970) or as an important creative evolutionary force (Anderson & Stebbins 1954; Ellstrand & Schierenbeck 2000). On the detrimental side, hybrid breakdown, or outbreeding depression, can decrease performance of hybrid individuals across a suite of traits linked to fitness, such as development time, mortality, and fecundity (Burton *et al.* 1999; Edmands 2002). On the positive side, hybridization can increase fitness relative to parents directly through heterozygote advantage (overdominance of beneficial traits) (Edmands 2002; Hedrick & Garcia-Dorado 2016; Lee *et al.* 2016) or by alleviating high mutational load (heterosis) and reducing inbreeding depression, and indirectly through restoring genetic variation lost through genetic drift or bottlenecks in population size (genetic rescue). Even in populations that have not experienced strong drift or bottlenecks, hybridization can increase overall population genetic variation, resulting in increased ability to respond to selection pressures (Fisher 1930). Additionally, hybridization can facilitate the formation of novel genotypes, potentially producing 'transgressive' phenotypes that fall outside the range of either parent (Rieseberg *et al.* 1999). Alternatively, hybridization can in some cases have minimal effects if the genetic distance between parents is small (Mallet 2005).

Intraspecific hybridization between recently diverged species may be particularly beneficial in colonizing populations that pass through strong bottlenecks in population size, in turn losing genetic variation, and potentially becoming inbred (Ellstrand & Schierenbeck 2000; Dlugosch & Parker 2008; Rius & Darling 2014; Laugier *et al.* 2016). In the planned release of specialized biological control agents, the goal is for the intentionally released population to

83 establish and propagate (Seastedt 2015), to feed on the target host (typically an invasive weed or
84 insect), and not shift to use other, non-target hosts. Biological control programs have a fairly low
85 success rate (<50%), mostly due to lack of establishment of agents in their new environment
86 (Van Driesche *et al.* 2010). As an evolutionary mechanism, hybridization might allow these
87 establishing populations to better face adaptive challenges in the novel environment. There is
88 some evidence that releasing different "strains" or ecotypes of biological control agents in an
89 effort to increase genetic variation might improve establishment success (Hopper *et al.* 1993;
90 Henry *et al.* 2010). New evidence suggests that increased genetic variation can be even more
91 important than augmenting population size in promoting population growth (Frankham 2015;
92 Hufbauer *et al.* 2015; Frankham 2016). Colonizing populations also experience novel
93 environments in which transgressive phenotypes may, by chance, have higher fitness than
94 parental phenotypes (Ellstrand & Schierenbeck 2000). Yet, the quantification of genetic variation
95 in populations of biological control agents planned for release is not yet a standard procedure,
96 likely because of the lack of studies investigating the effects of increased variation on long-term
97 establishment.

98 Releasing genetically distinct ecotypes in the same area can promote hybridization. Only
99 a few studies have looked at hybridization in biological control agents (Hoffmann *et al.* 2002;
100 Mathenge *et al.* 2010; Benvenuto *et al.* 2012; Szucs *et al.* 2012). Szucs *et al.* (2012) found that
101 hybridization improved performance in vital life-history traits, which could improve control of
102 the target pest. However, there is also evidence that hybridization can decrease host specificity or
103 increase host range as a result of changes in phenotype (Hoffmann *et al.* 2002; Mathenge *et al.*
104 2010). Such a change would increase the risk of biological control to non-target species
105 dramatically. Thus, it is imperative that more experiments are executed to understand the

106 consequences of hybridization for biological control programs, including evaluating the degree
107 to which it will be possible to draw general conclusions versus research being needed on a case-
108 by-case basis.

109 We present research in which we quantify the effects of hybridization between biological
110 control agents in the genus *Diorhabda* that were released to control *Tamarix* (saltcedar, or
111 tamarisk) in North America. Saltcedar in North America is comprised of a hybrid swarm of
112 *Tamarix chinensis* and *T. ramosissima* (Gaskin and Schall 2002). It is an invasive weed that has
113 colonized riparian habitats from Montana to Mexico (Gaskin & Schaal 2002). In 2001, the
114 USDA's Animal and Plant Health Inspection Service (USDA APHIS) approved the open field
115 release of the central Asian salt cedar leaf beetle, *D. elongata*, as a biological control agent for
116 saltcedar (DeLoach et al. 2003). *Diorhabda elongata* was classified as a dingle wide-ranging
117 species that specialized on *Tamarix* spp and comprised different subspecies and ecotypes. To
118 match environmental conditions in North America, the saltcedar biological control program
119 eventually utilized seven *Diorhabda* ecotypes with native ranges stretching from north Africa to
120 central Asia (Tracy & Robbins 2009; Bean *et al.* 2013a). A recent taxonomic revision of the
121 *Tamarix*-feeding *Diorhabda* has used morphological and biogeographical data to define this
122 group as a complex comprising five species: *D. meridionalis*, *D. carinulata*, *D. carinata*, and *D.*
123 *sublineata* (Tracy & Robbins 2009). Recent genetic studies using amplified fragment length
124 polymorphisms (AFLPs) revealed four major clades within this group which coincide with the
125 four morphospecies (Bean *et al.* 2013b). There was also a fifth species, *D. meridionalis*, not
126 currently used in the saltcedar biological control program. Currently, *D. carinulata* is the most
127 widespread of the species in North America and covers large areas in Oregon, Idaho, Wyoming,
128 Colorado, Utah, Nevada, northern Arizona, and northern New Mexico (Bean *et al.* 2013a). The

129 other three species have all been released in Texas and have started spreading (Michels *et al.*
130 2013). Hybridization is possible between all four taxa, but hybrids between *D. carinulata* and
131 each of the other three species produce few viable offspring. In contrast, egg viability of hybrids
132 between *D. elongata*, *D. carinata*, and *D. sublineata* is comparable to that of the parents (Bean *et*
133 *al.* 2013b). We crossed these three species reciprocally, and tracked performance over three
134 generations to quantify the effects of hybridization. We measured several life history traits
135 crucial to fitness, as well as host preference for saltcedar relative to a non-target plant, *Tamarix*
136 *aphylla* (athel hereafter) for both hybrid offspring and the parental species.

137 ## 2. Materials & Methods
138
139 ### *2.1 Organism*

140 The beetles used in our experiments stemmed from samples originally collected from
141 saltcedar in Eurasia and North Africa. Descendants of these samples were used to establish
142 laboratory populations maintained at the Palisade Insectary, Biological Pest Control Program,
143 Colorado Department of Agriculture, Palisade, CO (CDA Palisade). Colonies were maintained
144 on cuttings of saltcedar, including *T. ramosissima*, *T. chinensis*, and their hybrids (Gaskin &
145 Schaal 2002), in 7.5 liter capacity plastic containers with mesh siding for ventilation in
146 incubators under a light regime of 16:8 and 27°C/16°C. *Diorhabda carinata* ("C" hereafter) used
147 in this study were originally collected in 2002 near Karshi (Qarshi), Uzbekistan (38.86 N, 65.72
148 E; elevation 350 m), and *Diorhabda sublineata* ("S" hereafter) originated near the town of Sfax,
149 Tunisia (34.66 N, 10.67 E, elevation 10 m). Both these species were maintained in the lab prior
150 to our experiments. *Diorhabda elongata* ("E" hereafter) were collected from Sfakaki, Crete,
151 Greece (35.83 N, 24.6 E, elevation 7 m) and in 2004 they were first released upstream of
152 Esparto, CA along Cache Creek in the Capay Valley. Unlike the other two species, *D. elongata*

used in our experiments were collected in 2015 from the field in the Capay Valley and used to start a laboratory colony. No other species were released into the Capay Valley nor have any been established within 150 miles, and therefore it is reasonable to assume that there was no chance for hybridization before our experiments.

2.2 Crosses

To produce the first generation of hybrids, seven virgin females and seven males of each species were placed together into a plastic bucket with mesh siding (7.5 liter) with saltcedar. Since male female directionality can affect the fitness of hybrid offspring (Payseur & Rieseberg 2016), we crossed each species reciprocally. We thus made the following hybrids: *D. carinulata* x *D. elongata* ($C_f \times E_m$, $E_f \times C_m$), *D. carinulata* x *D. sublineata* ($C_f \times S_m$, $S_f \times C_m$), *D. elongata* x *D. sublineata* ($E_f \times S_m$, $S_f \times E_m$), plus the parents ($C_f \times C_m$, $E_f \times E_m$, $S_f \times S_m$,). To keep inbreeding depression to a minimum, we initiated two separate buckets for each of the parental lines so that density remained the same but so the parental generation had 14 families rather than 7 for the crosses. All adults were allowed to remain in the buckets for five days of egg-laying.

2.3 F_1 adult performance test

We counted the number of eggs produced over 48 hours as an estimate of performance of first generation hybrids. Buckets were checked daily for emergence of F_1 adults. On the day of emergence, adults were sexed and mating pairs were placed into a plastic container (0.4L) with a paper towel lining the bottom and food. The containers were checked daily for eggs. The number of eggs produced was counted for 48 hours after the first eggs were laid. After this time, F_1 adults were removed and killed by freezing.

2.4 F_2 larval performance test

We measured percent hatching of all eggs laid in the first 48 hours, development time (in days), and adult mass (mg) attained by each F_2 larva. Upon emergence, the date was recorded as well as the number of eggs that successfully hatched. Counting eggs is challenging due to the three-dimensional nature of the egg clutches. Following (Bean *et al.* 2013b), to ensure accuracy we also counted the number of larvae and compared this with the number of eggs. If the number of eggs was less than the number of larvae, we used the number of larvae as the total number of eggs produced. If the number of eggs was greater than the number of larvae, we conducted a recount of the clutch. Out of the hatched larvae from each mating pair, up to five were randomly chosen and allowed to develop individually.

Larvae were maintained in small plastic cups (0.4L) and given fresh saltcedar with its stem in a water-filled 1.5mL eppendorf tube each day. A paper-towel lined the bottom of each cup. When the larvae reached their last stage of development, 2 cm of sand was placed in each cup to provide conditions favorable for pupation. All larvae were maintained in incubators under a light regime of 16:8 (L:D) and 27°C/16°C, and rotated every other day to standardize environmental effects.

2.5 F_2 adult preference test

We conducted a host preference test to determine if hybridization affected host preference for the non-target species, athel, presenting beetles with a choice between saltcedar and athel. Athel is an ornamental that is found at more southern latitudes in the US and is considered invasive in the southwestern U.S. (Gaskin & Shafroth 2005). *Tamarix* hybrids of *T. ramosissima* and *T. chinensis* (saltcedar) and are considered the preferred field host of *Diorhabda*. Previous host testing showed that the three *Diorhabda* species used in this study can survive as well on athel as on saltcedar, will oviposit on either saltcedar or athel under laboratory

198 no-choice conditions, and showed an inconsistent preference for saltcedar under choice
199 conditions (Milbrath & Deloach 2006a; Milbrath & DeLoach 2006b). However, saltcedar is
200 preferred at field sites (Moran *et al.* 2009), and the intrinsic rate of increase of beetle populations
201 is reduced on athel due to smaller egg mass size and a delayed start to oviposition (Milbrath &
202 DeLoach 2006b).

203 Between 24 and 48 hours after emergence, we sexed and weighed the F_2 adults. The
204 beetles were placed in a plastic tub (3L) with two eppendorf tubes containing equal amounts of
205 either athel or saltcedar. Each beetle was placed in the middle of the tub, with both plants placed
206 equidistantly at 10 cm from the center. The beetle remained in the plastic tub for 24 hours, at
207 which time the amount of frass under each plant was weighed to the nearest 0.1 g (DeLoach *et*
208 *al.* 2003).

209 *2.6 F_3 larval performance test on two different hosts*

210 We measured F_3 larval performance on athel and saltcedar. After the host-choice test,
211 mating pairs were formed with F_2 adults from the same cross. All F_2 adults were given saltcedar
212 foliage to feed on regardless of what they chose as their host in the adult preference test. They
213 were placed in the same plastic dish as previously described and allowed to mate and oviposit.
214 The date of first oviposition, the number of eggs laid in 48 hours, and the percent hatching was
215 recorded. Larvae from each mating pair were split and a maximum of five larvae were placed in
216 a plastic dish with either athel or saltcedar. We measured development time to adult and adult
217 mass.

218 *2.7 Statistical analysis*
219
220 Our interests center on comparing the fitness of hybrids to their parental species. Thus,
221 each analysis was done separately for each of the 7 pairs of parental species and their respective

two hybrid crosses (male/female reciprocal). All statistical analysis was conducted using R version 3.3.2 (R_Core_Team 2016). For the first generation, we analyzed differences in the total number of eggs produced between hybrids and parental species using a standard linear model. The number of eggs was log-transformed to meet the assumption of homogeneity of variance. Percent hatching was analyzed as a proportion of hatched eggs compared to the total number of eggs using a generalized linear model with quasibionmial error distribution. Cross was the only fixed effect for number of eggs produced and percent hatching in the first-generation analysis. For the second and third generations, we quantified the development time from egg to adult (days), adult mass (mg), and host choice. For development time and adult weight, we used linear mixed-effects models through the lme4 package (Bates *et al.* 2015) with cross, sex and their interaction as fixed effects, and family as a random effect. For host choice with a binary response (saltcedar or athel), we used a generalized linear mixed effects model with a binomial error distribution. For the third generation, we also included the random effect of cup nested within family for development time and adult weight.

3. Results

3.1 Egg count, percent hatching

Hybridization did not significantly affect the number of F_1 eggs laid in 48 hours for any of the crosses (Tables 1-3). Cross had a marginally significant influence on percent of eggs that hatched with the E × E and $E_f \times S_m$ cross producing slightly fewer viable eggs than the other crosses ($F_{3,37} = 2.82$, $P = 0.052$, Table 2). In the F_2, only for the S × C cross was there a significant effect of cross on the number of eggs laid in 48 hours, where hybrids produced significantly more eggs than either parental species ($F_{3, 39} = 2.97$, $P = 0.044$, Table 1, Figure 1).

Cross did not affect the percentage of eggs hatched for any crosses in the second generation (Table 1-3).

3.2 Development time, adult mass

In the S × C crosses, females were larger (effect of sex: $\chi^2 = 12.98$, df = 1, $P < 0.001$, effect of cross: $\chi^2 = 16.39$, df = 3, $P < 0.001$) and hybrids developed faster (effect of sex: $\chi^2 = 9.93$, df = 1, $P = 0.002$, effect of cross: $\chi^2 = 20.60$, df = 3, $P<0.001$) (Table 1, 4, Figure 2). For the S × E cross, there was a significant interaction between sex and cross in development time, in that males developed slower than females in the S × S line (interaction cross*sex: $\chi^2 = 8.90$, df = 3, $P = 0.031$, Tables 2, 4). We also found a significant effect of sex and cross on adult mass in the S × E cross, with females being overall larger (effect of sex: $\chi^2 = 7.28$, df = 1, $P = 0.007$, effect of cross: $\chi^2 = 17.50$, df = 3, $P < 0.001$, Tables 2, 4). There was no effect of sex or cross on development time or adult mass for the E × C cross, although overall, females tended to be larger.

For the third generation, we were only able to investigate the effects of hybridization for the S × C cross (*D. sublineata* x *D. carinata*) due to limitations in the availability of our host plants. Development time was significantly affected by host plant and by cross, with both parents and hybrids developing slower on athel (effect of cross: $\chi^2 = 9.74$, df = 4, $P = 0.029$; effect of plant: $\chi^2 = 10.22$, df = 1, $P = 0.001$, Tables 1, 5, Figure 3). While there is a trend for hybrids to develop slower than parents regardless of host plant, there was no significant decrease in development time in hybrids compared to parents (effect of cross: Hybrids develop slower than the parents regardless of host plant, yet contrasts between crosses were not significant. Adult weight was not affected by hybridization, however females were larger regardless of cross (effect of sex: $\chi^2 = 10.124$, df = 1, $P = 0.001$, Tables 1, 5).

3.3 Host choice

We tested the host preference of individuals from all crosses in the second generation. Due to limitations in our host plant resources and, because of differences seen in the second generation, we also examined host preference for the S × C cross in the third generation. Sex did not affect host choice for any of the crosses in the second generation (Tables 1-3). Cross significantly affected host choice in the S × C cross (effect of cross: $\chi^2 = 9.87$, df = 3, $P = 0.031$) and S × E cross (effect of cross ($\chi^2 = 9.23$, df = 3, $P = 0.026$), whereas there was no difference in host preference between hybrids and their parents in the E × C cross (Tables 1-4, Figure 4). There was no effect of hybridization on host preference in the S × C cross in the third generation (effect of cross: $\chi^2 = 1.163$, df = 3, $P = 0.7619$, Tables 1, 5).

4. Discussion

In introduced species, the effects of hybridization can influence local adaption and determine the fate of colonization success and establishment (Rius & Darling 2014). Introduced biological control agents undergo similar pressures as newly invading species, and understanding the mechanisms behind population growth and establishment are crucial to the implementation of successful biological control. In this study, we investigated the effects of hybridization on various life history traits and host preference for three different species of the biological control agent *Diorhabda*. We confirmed that all three species were reproductively compatible (Bean *et al.* 2013b), and that found that reciprocal crosses produced viable offspring through at least two generations. Life history traits beyond the production of viable eggs were either unchanged or improved with hybridization when compared to the parental species. These results support the hypothesis that these species have not experienced reproductive isolation for long enough to allow the evolution of genetic incompatibilities.

293 Hybridization can have positive, neutral, or negative effects on fitness. These effects
294 depend on the genetic distance between mixing populations and the interactions between genes
295 and environment. Hybrid vigor is commonly seen in the first generation of admixture between
296 genetically distinct populations, and is typically thought to be due to masking of deleterious
297 alleles rather than overdominance (Szulkin *et al.* 2010), whereas hybrid breakdown is commonly
298 seen in the second or later generations due to recombination of the parental genes, allowing for
299 the possibility of deleterious allele combinations (heterozygote disadvantage) (Dobzhansky
300 1950; Edmands 2002). In our study, there was no difference between parents and their hybrid
301 offspring in fecundity or percent hatching in the first generation in any cross. Previous molecular
302 work done by Bean *et al* (2013b) showed that while all four *Diorhabda* species separated into
303 their own clades, the three species examined here were likely more closely related to each other
304 than to the congeneric *D. carinulata*. It is possible that these species are not genetically distinct
305 enough to be detrimentally affected by hybridization. However, the beetles used in our study had
306 been lab reared for varying amounts of time (at least ten generations), and may have become
307 inbred or lost variation via drift, both of which could reduce fitness. Thus, an alternative
308 explanation is that positive effects of crossing, via masking of deleterious mutations could have
309 balanced out potentially negative effects of hybridization, leading to zero, or close to zero, net
310 change in life history traits. The masking of deleterious mutations can persist for many
311 generations (Frankham 2016; Hedrick & Garcia-Dorado 2016), and thus further study
312 investigating the effects of hybridization for more than three generations would increase our
313 understanding.

314 Our results show that some of our crosses benefited greatly from hybridization in
315 fecundity and development time in the second generation, and thus we see no evidence of hybrid

316 breakdown. S × C crosses produced 67% more eggs and developed approximately 7 days shorter
317 than the parental species. The E × C cross exhibit the same trend, although this was only
318 marginally significant. Other crosses showed no effect of hybridization, and none of our crosses
319 suffered a fitness cost. In the S × C cross, where we could examine a third generation, we saw no
320 effect of hybridization on fecundity, but we did see a trend that hybrids were developing slower
321 on both host plants. For this analysis, our sample size was lower than for the previous
322 generations, and so further work is necessary to determine if development time slowed because
323 of hybridization.

324 Changes in host specificity in a released agent are one of the most concerning issues to
325 scientists studying biological control (Van Klinken & Edwards 2002; Brodeur 2012; McEvoy *et*
326 *al.* 2012). Our results show that host specificity can indeed be affected by hybridization, and that
327 the phenotype can vary depending on the maternal or paternal species. In the S × C crosses, host
328 preference of the hybrid followed the preference of the maternal species, whereas in the S × E
329 cross, hybrids showed no preference for either host plant where the parents both showed a strong
330 preference for the target host. Host specificity depends upon a suite of traits, such as behavior,
331 morphology, and life-history strategies and as such is highly constrained (Zwolfer & Harris
332 1971; Giebink *et al.* 1984; Chang *et al.* 1987). Even so, in more generalist species than are
333 typically used for biological control, host use has been shown to have a genetic basis, and can
334 thus vary between individuals and populations (Singer & Parmesan 1993; Funk 1998). In our
335 study, the inherited pattern for host use depended not only on the cross, but the preference of the
336 maternal species. A growing body of literature suggests that for herbivorous insect species,
337 mothers have been shown to influence host use (Amarillo-Suarez & Fox 2006; Egan *et al.* 2011;
338 Cahenzli & Erhardt 2013). Egan *et al.* (2011) specifically demonstrated that host-use and

performance are traits with sex-linked maternal influence. Consequently, the pattern of host specificity in hybrid crosses can be hard to predict since it will depend not only on the amount of genetic variation across a suite of traits, but also parental influence.

Using hybridization in biological control presents unique challenges. On one hand, increased genetic variation, potentially from hybridization, can buffer introduced populations against adaptive challenges and thus increase the probability of establishment and effective control (Hopper *et al.* 1993). On the other, the genetic admixture of previously isolated populations might give rise to new phenotypes that are less desirable, such as a change in host specificity (Hoffmann *et al.* 2002; Mathenge *et al.* 2010). Our results demonstrate that while some crosses benefit from hybridization in terms of development time and fecundity, differences in host specificity due to hybridization is of concern.

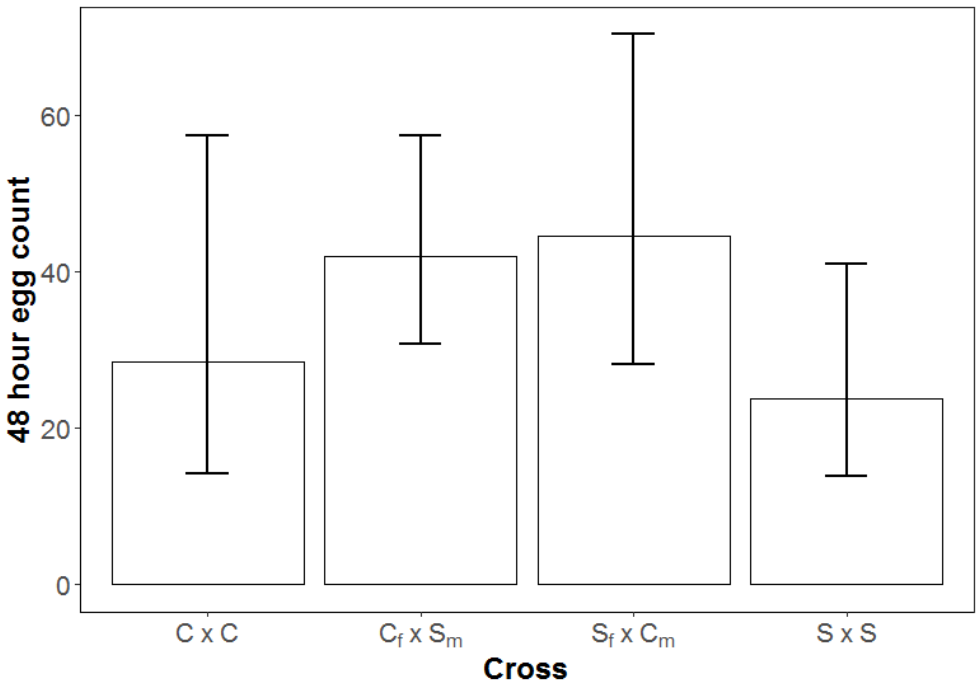

Figure 1: Number of eggs produced in first 48 hours by *D. carinata* (C × C), *D. sublineata* (S × S), and their hybrids in the second generation. Cross significantly affected egg production, with hybrids producing more than either parental species. Bars represent 95% confidence intervals.

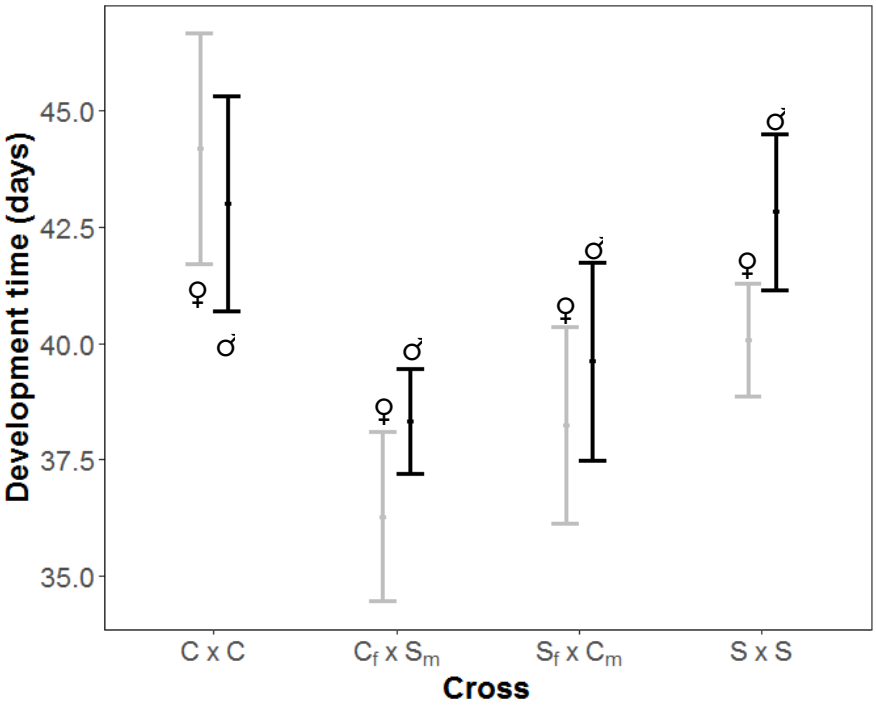

Figure 2: Development time from hatching until adult for each sex of *D. carinata* (C × C), *D. sublineata* (S × S) and their hybrids in the second generation. Cross significantly affected development time, with hybrids developing faster than either parental species. Grey and black lines represent females and males, respectively. Bars represent 95% confidence intervals.

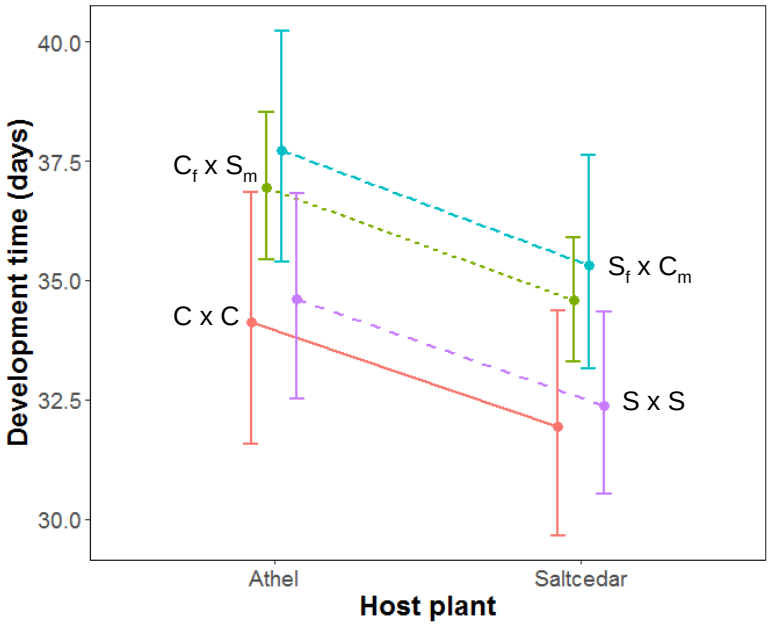

Figure 3: Development time on athel (non-target) and saltcedar (target) for *D. carinata* (C × C), *D. sublineata* (S × S), and their hybrids after three generations of hybridization. $S_f \times C_m$ are long close dashes, $C_f \times S_m$ small dashes, S × S long spaced dashes, and C × C solid line. Host plant significantly affected development time with beetles developing slower on the non-target host.

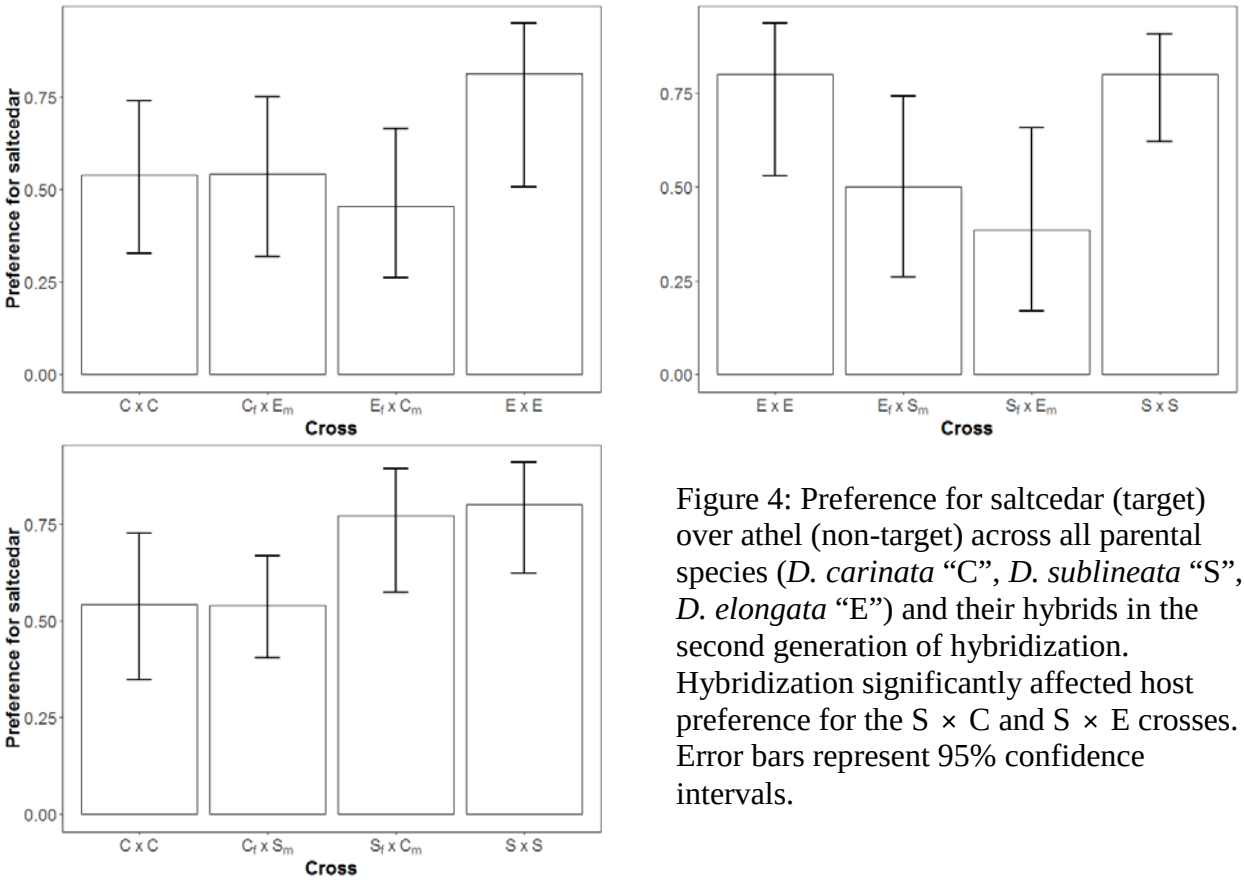

Figure 4: Preference for saltcedar (target) over athel (non-target) across all parental species (*D. carinata* "C", *D. sublineata* "S", *D. elongata* "E") and their hybrids in the second generation of hybridization. Hybridization significantly affected host preference for the S × C and S × E crosses. Error bars represent 95% confidence intervals.

Table 1: Trait means (95% CI) for each generation of the *D. sublineata* by *D. carinata* cross. Letters indicate significant differences between crosses.

Gen	Trait		Cross: C × C	$C_f \times S_m$	$S_f \times C_m$	S × S	F value	*P*
1	48 hour egg count		25.53 (20.08, 32.46) [a]	21.54 (17.13, 27.08)[a]	30.14 (22.38, 40.59)[a]	20.25 (15.73, 26.08)[a]	$F_{3, 69} = 1.733$	0.1682
		N	20	22	13	18		
	Proportion hatching		91.4% (66.0, 99.0)[a]	90.9% (68.0, 98.6)[a]	91.8% (59.9, 99.6)[a]	94.3% (67.2, 99.8)[a]	$F_{3, 60} = 0.8425$	0.476
		N	17	20	12	15		
2	Dev time (days) (male)		43.6 (41.2, 45.9)[a]	38.2 (36.3, 40.1)[b]	39.6 (37.4, 41.9)[ab]	43.5 (41.0, 45.9)[a]	See Table 4 for statistical results	
		N	14	31	18	17		
	Dev time (days) (female)		44.1 (41.6, 46.7)[a]	36.2 (34.3, 38.2)[b]	38.7 (36.2, 41.3)[b]	39.8 (37.6, 41.9)[b]		
		N	11	22	12	13		
	Adult mass (mg) (male)		21.0 (19.3, 22.8)[ab]	22.6 (21.2, 24.0)[a]	19.4 (18.0, 21.0)[b]	18.3 (16.9, 19.8)[b]		
		N	14	31	18	17		
	Adult mass (mg) (female)		22.5 (18.5, 27.5)[ab]	25.8 (22.2, 29.9)[a]	20.6 (16.9, 25.1)[ab]	19.2 (16.2, 22.8)[b]		
		N	11	22	12	13		
	Preference for saltcedar		54.2% (34.6, 72.5)[a]	53.8% (40.3, 66.8)[a]	76.9% (57.2, 89.2)[a]	80.0% (62.1, 90.7)[a]		
		N	24	52	26	30		
	48 hour egg count		25.0 (16.1, 38.8)[a]	41.7 (32.1, 54.1)[a]	45.6 (33.0, 63.1)[a]	27.0 (18.9, 38.7)[a]	**$F_{3, 39} = 2.9659$**	**0.0437**
		N	6	17	11	9		
	Proportion hatching		93.2% (26.5, 99.1)[a]	94.7% (67.7, 99.8)[a]	94.2% (50.0, 99.9)[a]	79.7% (29.7, 98.9)[a]	$F_{3, 26} = 1.3756$	0.2722
		N	3	15	7	5		
3	Dev time (days) saltcedar		31.9 (29.7, 34.3)[a]	34.6 (33.3, 35.9)[a]	35.3, (33.2, 37.6)[a]	32.4 (30.5, 34.3)[a]	See Table 5 for statistical results	
		N	9	38	9	12		
	Dev time (days) athel		34.1 (31.6, 36.9)[a]	37.0 (35.4, 38.6)[a]	37.7 (35.4, 40.2)[a]	34.6 (32.5, 36.9)[a]		
		N	3	24	11	7		
	Adult mass (mg) (male)		19.5 (15.9, 23.9)[a]	19.7 (17.7, 21.8)[a]	18.6 (15.8, 22.0)[a]	16.7 (14.1, 19.7)[a]		
		N	9	38	9	12		
	Adult mass (mg) (female)		22.5 (18.4, 27.6)[a]	22.8 (20.6, 25.3)[a]	21.6 (18.1, 25.6)[a]	19.3 (16.4, 22.7)[a]		
		N	3	24	11	7		
	Preference for saltcedar		69.0% (37.2, 89.3)[a]	61.8% (47.3, 74.5)[a]	50.2% (30.3, 75.7)[a]	71.4% (45.6, 88.1)[a]		
		N	12	62	20	19		

Table 2: Trait means (95% CI) for each generation of the *D. sublineata* by *D. elongata* cross. Letters indicate significant differences between crosses.

Gen	Trait		Cross: S × S	Cross: $S_f \times E_m$	Cross: $E_f \times S_m$	Cross: E × E	F value	*P*
1	48 hour egg count		20.25 (15.45, 26.55)[a]	22.46 (16.12, 31.28)[a]	26.24 (18.84, 36.55)[a]	18.04 (10.16, 32.03)[a]		
		N	18	12	12	4	$F_{3, 42} = 0.4807$	0.5706
	Proportion hatching		94.3% (67.2, 99.8)[a]	90.6% (58.5, 99.3)[a]	87.62% (51.6, 98.9)[a]	87.8% (30.2, 99.9)[a]		
		N	15	12	10	4	**$F_{3, 37} = 2.8245$**	**0.05193**
2	Dev time (days) (male)		43.4 (40.8, 46.2)[a]	37.5 (34.7, 40.5)[b]	39.5 (35.5, 44.0)[ab]	42.2 (38.6, 46.3)[ab]	See Table 4 for statistical results	
		N	17	9	5	13		
	Dev time (days) (female)		39.8 (37.8, 42.0)[a]	38.0 (35.2, 41.0)[a]	42.4 (39.7, 45.3)[a]	43.3 (39.2, 47.7)[a]		
		N	13	5	10	3		
	Adult mass (mg) (male)		18.1 (16.2, 20.2)[a]	13.1 (11.5, 15.1)[b]	17.1 (14.1, 20.8)[ab]	17.0 (14.6, 19.9)[ab]		
		N	17	9	5	13		
	Adult mass (mg) (female)		19.1 (16.7, 21.9)[a]	15.6 (12.8, 19.0)[a]	21.5 (18.1, 25.5)[a]	18.8 (14.5, 24.3)[a]		
		N	13	5	10	3		
	Preference for *Tamarix* spp		80.0% (62.1, 90.7)[a]	38.5% (17.0, 65.6)[b]	50.0% (26.0, 74.0)[ab]	80.0 (53.0, 93.4)[ab]		
		N	30	13	14	15		
	48 hour egg count		27.0 (17.7, 41.2)[a]	27.0 (14.4, 50.9)[a]	45.1 (21.8, 93.6)[a]	28.0 (11.4, 68.3)[a]	$F_{3, 14} = 0.622$	0.6124
		N	9	4	3	2		
	Proportion hatching		79.7% (29.7, 98.9)[a]	70.5% (14.2, 98.9)[a]	96.6% (18.1, 96.7)[a]	94.1% (4.3, 92.5)[a]	$F_{3, 7} = 0.9465$	0.4682
		N	5	3	2	1		

Table 3: Trait means (95% CI) for each generation of the *D. elongata* by *D. carinata* cross. Letters indicate significant differences between crosses.

Gen	Trait		Cross: E × E	$E_f \times C_m$	$C_f \times E_m$	C × C	F Value	*P*
1	48 hour egg count		18.04 (9.93,32.77)[a]	18.90 (14.37, 24.85)[a]	18.36 (13.96, 24.14)[a]	25.53 (19.55, 33.35)[a]		
		N	4	19	19	20	$F_{3, 58} = 1.3855$	0.2837
	Proportion hatching		87.8% (35.1, 99.4)[a]	86.8% (58.1, 99.7)[a]	90.7% (61.9, 99.1)[a]	91.4% (66.0, 99.0)[a]		
		N	4	11	14	17	$F_{3, 42} = 0.7341$	0.5376
2	Dev time (days) (male)		42.4 (38.4, 46.3)[a]	39.5 (36.6, 42.4)[a]	42.5 (39.7, 45.4)[a]	43.6 (41.1, 46.3.4)[a]	See Table 4 for statistical results	
		N	13	12	18	14		
	Dev time (days) (female)		43.33 (38.57, 48.09)[a]	39.13 (36.55, 41.71)[a]	40.0 (36.3, 43.7)[a]	44.0 (41.02, 47.17)[a]		
		N	3	14	5	11		
	Adult mass (mg) (male)		17.1 (14.9, 19.6)[a]	18.8 (16.7, 21.0)[a]	20.5 (18.4, 22.9)[a]	21.0 (19.0, 23.3)[a]		
		N	13	12	18	14		
	Adult mass (mg) (female)		18.8 (13.7, 25.8)[a]	22.1 (18.7, 26.1)[a]	19.7 (15.4, 25.2)[a]	22.7 (18.7, 27.5)[a]		
		N	3	14	5	11		
	Preference for *Tamarix* spp.		81.5% (50.8, 94.9)[a]	45.6% (26.2, 66.4)[a]	54.3% (31.9, 75.1)[a]	54.1% (32.8, 74.0)[a]		
		N	15	26	22	24		
	48 hour egg count		28.0 (15.0, 52.0)[a]	35.6 (27.9, 45.4)[a]	50.1 (33.8, 74.1)[a]	25.0 (17.5, 35.8)[a]	$F_{3, 22} = 2.6441$	0.07446
		N	2	13	5	6		
	Proportion hatching		94.1% (2.0, 97.2)[a]	74.6% (58.6, 99.9)[a]	93.0% (35.3, 99.3)[a]	93.2% (26.5, 99.1)[a]	$F_{3, 14} = 1.5797$	0.2387
		N	1	10	4	3		

Table 4: Results from generalized linear mixed-effects models for the second generation of hybridization for all crosses.

	S × E Cross			**Random effects**			
	Cross	Sex	Cross*sex	Family		Residual	
Trait	$\chi 2$, (df), *P*	$\chi 2$, (df), *P*	$\chi 2$, (df), *P*	Variance	Std dev	Variance	Std dev
Dev time	**10.82, (3), 0.013**	0.47, (1), 0.493	**8.90, (3), 0.031**	0.006174	0.07857	0.001771	0.04208
Adult mass	**17.50, (3), <0.001**	**7.28, (1), 0.007**	2.56, (3), 0.464	0.01266	0.1125	0.02347	0.1532
Preference for saltcedar	**9.23, (3), 0.026**	0.98, (1), 0.322	0.99, (3), 0.804	0	0	0	0
	C × E Cross						
Dev time	5.99, (3), 0.112	0.08 (1), 0.7837	5.79, (3), 0.122	0.008246	0.09081	0.001845	0.04295
Adult mass	5.68, (3), 0.128	3.47, (1), 0.0626	2.65, (3), 0.449	0.01058	0.1029	0.0348	0.1868
Preference for saltcedar	3.66, (3), 0.300	0.89, (1), 0.3464	3.69, (3), 0.297	0.6548	0.8092	0	0
	S × C Cross						
Dev time	**20.60, (3), <0.001**	**9.934, (1) 0.002**	5.82, (3), 0.120	9.787	3.128	4.205	2.051
Adult mass	**16.39, (3), <0.001**	**12.982, (1), <0.001**	2.73, (3), 0.435	0.02041	0.1429	0.02266	0.1505
Preference for saltcedar	**9.87, (3), 0.031**	0.3953, (1), 0.5295	1.89, (3), 0.596	0	0	0	0

Table 5: Results from generalized linear mixed-effects models for the third generation of the *D. sublineata* by *D. carinata* cross.

	S × C Cross				**Random effects**					
	Cross	Sex	Plant	Plant * Cross	Cup within family		Family		Residual	
Trait	$\chi 2$, (df), *P*	$\chi 2$, (df), *P*	$\chi 2$, (df), *P*	$\chi 2$, (df), *P*	Variance	Std dev	Variance	Std dev	Variance	Std dev
Dev time	**9.74, (3), 0.030**	0.022, (1), 0.882	**10.22, (1), 0.001**	3.46, (3), 0.326	0.0027	0.052	0.0009	0.0303	0.00305	0.05531
Adult mass	4.16, (3), 0.244	**9.74, (1), 0.021**	1.39, (1), 0.239	2.67, (3), 0.449	0.02133	0.1461	0	0	0.04805	0.2192
Preference for saltcedar	1.16, (3), 0.762	0.12, (1), 0.728	1.00, (1), 0.317	0.30, (3), 0.960	0	0	0	0	0	0

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