The Effect of Altered Soil Moisture on Hybridization Rate in a Crop-Wild System (*Raphanus* spp.)

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Abstract

Since plant mating choices are flexible and responsive to the environment, rates of spontaneous hybridization may vary across ecological clines. Developing a robust and predictive framework for rates of plant gene flow requires assessing the role of environmental sensitivity on plant reproductive traits, relative abundance, and pollen vectors. Therefore, across a soil moisture gradient, we quantified pollinator movement, life-history trait variation, and unidirectional hybridization rates from crop (*Raphanus sativus*) to wild (*Raphanus raphanistrum*) radish populations. Both radish species were grown together in relatively dry (no rain), relatively wet (double rain), or control soil moisture conditions in Ohio, USA. We measured wild and crop radish life-history, phenology and pollinator visitation patterns. To quantify hybridization rates from crop-to-wild species, we used a simply inherited morphological marker to detect F₁ hybrid progeny. Although crop-to-wild hybridization did not respond to watering treatments, the abundance of hybrid offspring was higher in fruits produced late in the period of phenological overlap, when both species had roughly equal numbers of open flowers. Therefore, the timing of fruit production and its relationship to flowering overlap may be more important to hybrid zone formation in *Raphanus* spp. than soil moisture or pollen vector movements.

Introduction

As climate change transforms environments [1], it can profoundly affect biotic processes occurring within those environments, such as species coexistence, migration and evolutionary trajectories [2–4]. If mating processes are affected by environmental variation [5], this can have numerous and cascading effects on fecundity [6, 7], the organization of genetic diversity within versus among populations [8, 9], and, ultimately, population persistence [10]. For instance, plant populations with more genetic diversity may be able to more rapidly adapt in response to environmental change than those with less diversity. Of all plant traits, mating...
systems are perhaps the most influential in structuring genetic diversity within and among populations by both transmitting diversity across generations and determining rates of diversity loss [11]. Plant reproduction can be extremely labile. For instance, autogamous plants (those that self-fertilize) may possess relatively lower heterozygosity and allelic diversity, whereas allogamous plants (those that outcross) may have relatively higher heterozygosity and allelic diversity [11]. Thus, environmentally-induced changes in plant mating patterns may alter the organization of intra- and inter-specific genetic diversity and could further magnify or dampen biotic responses to anthropogenic climate change [12]. Therefore, to develop a robust and predictive framework for the science of mating systems and gene flow, it is critical to understand how plant mating systems respond to environmental variation.

Elevated atmospheric levels of CO$_2$ has increased the earth’s surface temperature [1, 13]. These climatic changes can strongly alter the availability of soil moisture to plants [13–18]. Most notably, changing climactic conditions have shifted geographic distributions by altering demography [19]. Researchers have repeatedly documented altered phenology and investment in reproductive structures in response to changing moisture regimes via the processes of adaptive evolution and phenotypic plasticity (e.g., [20–22]). These responses may impact the likelihood of interspecific hybridization, contribute new genetic material to recipient populations, generate novel phenotypes [23, 24], and initiate different evolutionary trajectories [25, 26]. Thus, hybridization rates may be indirectly influenced by environmental variation, including key abiotic variables influenced by global climate change.

Many studies of gene flow have attempted to identify key factors that contribute to successful hybridization (e.g., [27–29]). Similar to this study, most manipulate an experimental variable and measure the response of hybridization rate. However, it is critical to also examine the relationship between hybridization rate and traits that directly contribute to plant reproductive success and the probability of hybridization. For example, physical proximity, flowering phenology, investment in reproduction, and shared pollinators may partially drive hybridization rate to influence, at a small scale, the proportion of hybrid offspring, and, at a larger scale, the distribution and abundance of hybrid zones [27–35]. For plants, soil moisture can determine the size and abundance of flowers, and shift floral phenology [36, 37]. Further, hybridization rates depend on relative parental investment in male and female function—traits which commonly respond to water availability [38, 39]. Moreover, the number and size of flowers produced can alter pollinator communities and/or pollinator movement, which can influence the potential to hybridize [40–42]. For example, pollinators visit flowers with large corolla diameter (e.g., Collinsia parviflora [43]) or large numbers of flowers (e.g., Raphanus raphanistrum [44]) more frequently than plants with small corollas or few flowers. When floral display size of one or both species is affected by soil moisture, hybridization rates may shift in intensity (i.e., possibly causing swamping). Our recent work has documented that R. raphanistrum corolla diameter and flower production decreases with decreasing soil moisture [45, 46]. Thus, shifts in hybridization rates may be a consequence of a series of plant responses to local environmental conditions. Yet, despite the potential evolutionary consequences of hybridization, we know little about how environmental variation directly impacts mating systems, and thereby gene flow.

In this study, we used plants from experimental populations of the weedy annual R. raphanistrum and its cultivated relative (R. sativus) to investigate the effects of environmental variation (i.e., in soil moisture) on hybridization rates, as well as how these rates may be affected by local ecological context (i.e., neighbouring plants and insects responding to the same environmental variation). R. raphanistrum is a well-established model system in studies of plant evolution and ecology that has been used to evaluate the ecological consequences of crop-to-wild gene flow (e.g., [28, 47, 48]). However, few have investigated whether environmental variation
affects interspecific gene flow rates, especially among crops and their wild relatives (but see [35, 49]). We estimated the response of hybridization rate between wild and cultivated radish to moisture, as well as other plant traits using a manipulative field experiment. We also explored how hybridization rate could be predicted by plant traits using a regression approach. Thus, we asked the following questions:

- What is the hybridization rate between crops and their wild relative, and under which environmental conditions is the hybridization rate maximized?

- Does soil moisture affect hybridization rate by altering plant size or age at reproduction, floral synchrony, or pollinator movement between parental species?

A basic understanding of how the environment, specifically soil moisture, is likely to impact hybridization rates is imperative to assess potential ecological consequences of climatic variation and global change. We discuss the potential implications of climate change for interspecific hybridization with a special focus on the introgression of crop alleles into weed populations.

**Methods**

**Study Species**

Cultivated radish (*R. sativus*) is an annual, cosmopolitan root vegetable. Wild radish, or jointed charlock (*R. raphanistrum* L.), is a closely related species, differentiated from the cultivar by a chromosomal translocation and a suite of morphological characters [50, 51]. Although *R. raphanistrum* flowers earlier than *R. sativus*, their flowering phenologies overlap [47] and they share pollinators [52]. Therefore, when the two co-occur, they can spontaneously hybridize, often producing localized hybrid swarms [53]. Importantly, in the *Raphanus* crop-wild system, successful gene flow can be challenging and tends to occur in one direction only, with crop pollen fertilizing ovules on wild plants (i.e., crop-to-wild hybridization), for a number of reasons. First, fruits produced by crop plants remain attached to the maternal plant even after death, thus restricting seed dispersal of wild-to-crop hybrid plants to farm fields. Second, crop radishes are generally harvested before flowering making successful reproduction difficult for wild-to-crop hybrids. Nevertheless, crop plants are often allowed to flower before harvest in home gardens, which is the scenario we mimicked here.

As in Campbell et al. [54], we used flower colour, a simply inherited Mendelian trait, to distinguish hybrid from non-hybrid offspring. White flower colour stems from a dominant, crop-specific allele at this petal colour locus; yellow flower colour indicates a homozygous recessive genotype [50]. First-generation (F₁) hybrid offspring are easily distinguished as they invariably produce white petals when derived from a *R. raphanistrum* mother with yellow petals. As plants flowered, we recorded their flower petal colour, which allowed us to estimate the proportion of plants with white flowers (hybrids) compared to yellow flowers (non-hybrids) in each bulk sample.

**Seed Source**

The self-incompatible, *R. sativus* open-pollinated cultivar “Red Silk” (Harris-Moran Seed Co., Modesto, CA) acted as a cultivated pollen donor (homozygous for the dominant white flower petal colour allele). *R. raphanistrum* seeds, collected from a natural population close to Binghamton, New York, and grown in a common garden for several generations by the lab of Dr. J Conner in East Lansing, Michigan [55], acted as our maternal, weedy parent (homozygous for...
the recessive yellow flower petal colour allele). Given the self-incompatible mating system of *R. raphanistrum* and *R. sativus*, it was expected that these populations would be admixed and thus were representative of the genetic diversity occurring in both wild and crop populations.

**Experimental Design**

In 2010, we established 36 experimental plots containing *R. sativus* and *R. raphanistrum* in agricultural fields on Waterman Experimental Farm at The Ohio State University in Columbus, OH, USA (*N*<sub>sativus</sub> = 324, *N*<sub>raphanistrum</sub> = 324). The field experiment was planned as a split-split plot design with nine blocks. Four watering treatments (further described below), including (1) Control Unsheltered, (2) Control Sheltered, (3) No Rain, and (4) Double Rain, were randomly applied to 2.4 x 3m plots within each of the nine blocks.

The experimental layout was conceived of as a split-split plot design. For this design, each moisture treatment was applied on the large plots; the two radish species were planted separately into two smaller subplots, each including nine individuals (i.e., either nine wild or nine cultivated radishes). Then each species had data collected on it three times; thus, date of collection was considered a sub-sub plot factor.

Experimental plots were spaced at least 200 m apart. We imposed four watering treatments that significantly altered soil moisture:

1. Control Unsheltered (CU), where rainfall was not manipulated;
2. Control Sheltered (CS), where, to understand the effect of the shelter on plants (separate from the effect of altered rainfall), rain-exclusion shelters funneled collected water into a 227L barrel. The collected rainwater was then applied to the plot within 48 hours of the rain event. Therefore, CS plots received the same amount of rain that fell in CU plots.
3. No Rain (NR), where rain-exclusion shelters intercepted all rainfall (although rain could and did blow in from the side of the structure) and the water was collected in a barrel;
4. Double Rain (DR), where rain-exclusion shelters intercepted the rain, which collected in a barrel; this rainwater was then applied to the plot within 48 hours of the rain event (identical to CS plots). In addition, the rainwater collected in a neighboring NR plot was also applied, which effectively doubled the soil moisture within the plot.

The rain-exclusion shelters were 1.52m in height on the gutter edge and 2.44m on the high edge of the roof. As reported in Campbell et al. [45], water manipulation treatments significantly and predictably altered the average soil volumetric moisture content (%VMC) within each experimental plot. No rain plots were approximately 50% drier than CS plots whereas DR plots were approximately 50% wetter than CS plots [45]. Finally, the %VMC of CU and CS plots did not differ significantly in 2010. Finally, there was a significant interaction between watering treatment and sampling date where %VMC of CS and CU plots tended to decline significantly over the summer, whereas NR plots tended to remain relatively dry and DR plots tended to remain relatively wet.

Seeds were planted on May 15, 2010, grown in a greenhouse for two weeks, and then seedlings were transplanted into tilled plots. We transplanted nine *R. sativus* on the south-western side and nine *R. raphanistrum* on the south-eastern side of each plot. Plants were spaced 46 cm apart. To ensure transplant survival, water was applied to all seedlings. After the first week, the experimental watering treatments were implemented for the remainder of the experiment. When assessed, we measured soil moisture three times at the center of each plot, using a TDR (Field Scout, TDR 100/200 Spectrum Technologies, Inc., Plainfield, IL, USA) at 10 cm depths. Measurements were made after each redistribution of rainfall, i.e., eight times during the
growing season (between July 26, 2010 and September 28, 2010), and the plot-level estimate was averaged after these eight measurements. Weeds were removed when detected to ensure low levels of inter-specific competition and mimic garden plot conditions. However, this action may have also reduced inter-specific competition for floral insect visitors within the plot area (potentially increasing gene flow) or altered the attractiveness of plots to floral insect visitors (potentially reducing gene flow).

On the first day a plant flowered, we recorded plant age and stem diameter (an index of overall plant size). For each plot, the average days to first flowering and average stem diameter at first flowering was calculated for each species. On a weekly basis, we counted the number of open flowers on each plant within a plot. Across all weeks, the total number of flowers produced by all R. raphanistrum or all R. sativus plants within a plot was summed. The likelihood of hybridization is known to shift with the numerical advantage of plant ovules and pollen [56]. To estimate the relative availability of R. raphanistrum versus R. sativus hermaphroditic flowers, we calculated a weekly and overall index of relative flowering intensity (RFI) where the number of R. raphanistrum flowers was the numerator and the number of R. sativus flowers was the denominator. When RFI is greater than one, then R. raphanistrum flowers outnumber R. sativus flowers, and vice versa.

**Seed collection to estimate hybridization rate**

To measure hybridization rate, we waited until plants of both species were flowering in the majority of plots to begin collecting seeds and then collected seeds on three dates (the Timing factor in analyses described below). To identify when, throughout the reproductive season, hybridization was taking place we allowed the plants to flower in synchrony for two weeks and then tagged (with a coloured plastic zip tie) two incipient fruits per flowering R. raphanistrum plant on July15th, 2012 (Early-season fruits). Then, we waited an additional two weeks, and tagged two additional incipient fruits per flowering R. raphanistrum plant on July 29th, 2010 (Mid-season fruits). Finally, after another two-week period, we tagged two additional incipient fruits per flowering R. raphanistrum plant on August12th, 2010 (Late-season fruits). All tagged fruits, along with two additional fruits produced directly above and/or directly below the tagged fruits (to ensure a similar date of flower pollination), were collected as they ripened and before dehiscence. Therefore, for each plant, we collected a total of six fruits per week, for a maximum of 18 possible fruits over the course of the season. We then removed the seeds from their siliques and stored bulked seeds from fruit collected simultaneously.

**Insect visitation**

To assess the potential movement of R. sativus pollen to R. raphanistrum stigmas (i.e., movements that could have resulted in crop-wild hybridization), we monitored the movement of putative pollinators. Insect visits were monitored in each plot for 15 minute intervals between 8:00 and 16:00 h on spaced through the flowering season. We observed insect visitation activity for 88 of these 15 min intervals for a total of 22 h (n_C0 = 17, n_CS = 24, n_NR = 27, n_DR = 20). During that time, 4423 insect visits were recorded.

At the beginning and end of each observation interval, we recorded the number and type of flying insects in the plot (i.e., excluding ants and spiders) and followed the movement of haphazardly chosen flying insects. A potential pollination “visit” was recorded each time an insect landed on a flower. The visited plant species and individual experimental plant identity were also noted. This allowed us to assess the potential for pollen movement between- versus within-species where we assumed that movement from R. sativus to R. raphanistrum by insect visitors were potential hybridization events that could be measured in R. raphanistrum progeny. From
a potential pollination event, the type of insect was noted [57]. All insect visits were recorded during observational periods because visitation frequency was low. When an insect left the plot before the end of a given observational time period, we found another haphazardly chosen winged insect to monitor for movement until the 15-minute observation interval was over. From these data, we estimated the frequency of switching from *R. sativus* to *R. raphanistrum* (and not *vice versa*) by visiting flying insects, remembering that weedy plants in the *Raphanus* spp. complex are only generated when crop pollen fertilizes wild ovules. To explore whether pollinator movement responded to experimental watering treatments, we calculated the frequency of insect movement that could result in the production of hybrids on *R. raphanistrum* (i.e., from *R. sativus* to *R. raphanistrum*) relative to other possible movements (i.e., *R. sativus* to *R. sativus*, *R. raphanistrum* to *R. raphanistrum*, or *R. raphanistrum* to *R. sativus*).

**Common Garden to Assess Hybridization Rate**

Seeds produced in 2010 were moved from Ohio to Ontario when the Campbell Lab relocated to Ryerson University, Toronto, Ontario. In 2011, we measured the rate of hybridization from crop-to-wild radish using 2010 *R. raphanistrum* progeny in a common garden at Koffler Scientific Reserve (KSR) in King City, ON, Canada (Hill 44°01’N, 79°32’W, 285 masl). In late May, up to ten seeds from each sample collected in 2010 were planted in 20 mL of PRO-MIX BX peat within plastic seedling trays and placed in a greenhouse at KSR (The number of total genotyped plants is available in S1 Table). After developing their first true leaves, seedlings were transplanted directly into the ground at 30cm within a cleared and tilled garden area at KSR. Plants were watered to ensure transplantation success after which no additional watering treatments (as in 2010) were applied. Plants from each 2010 plot were randomly distributed within the garden and were randomly transplanted, in a grid-like fashion. Assays for flower colour as an indication of hybridization rate were not negatively affected by location bias because flower petal colour is insensitive to environmental conditions [50, 54]. To protect plants from insect herbivory, plants were sprayed with the insecticide Malathion (Loveland Products Canada, Inc., Dorchester, ON, Canada) on June 21, 2010. Aphids were present at low densities later in the season but did not colonize any plant heavily. We successfully genotyped 8110 plants while 756 plants died before being genotyped. For each 2010 plot, the frequency of hybrids produced on *R. raphanistrum* plants was calculated, as both the frequency of hybridization events (0/1) and number of hybrid offspring relative to the total number of offspring.

**Statistical Analysis**

We used PROC GLIMMIX in SAS Enterprise Guide (version 6.1) to employ generalized linear mixed models to predict the effect of watering treatments, species, time, and their interactions (as appropriate). For response variables we used frequency of hybridization events, frequency of hybrid offspring, days to flowering, stem diameter, overall abundance of flowers, relative flowering intensity (RFI), and pollinator movement. Our treatment factors were treated as fixed, while block and any interactions with block were deemed random. We used the option in Proc GLIMMIX to specify the distribution and corresponding LINK function of the data to improve model fit by choosing a distribution where the residuals best fit a normal distribution. As mentioned earlier, ours was a split-split plot design with watering treatment treated as a main plot factor, radish species as a subplot factor, and timing of fruit collection (i.e., early, mid or late) or the timing of moisture measurements as a sub-sub plot factor. However, analysis of some variables (e.g., hybridization rate or RFI) combined data for both species, so the timing factor were treated as a subplot factor (Table 1). Similarly, pollinator movement was simply tested for the effect of the watering treatment. Given the nature of the split-split plot
design where treatments are applied at different scales, we used different errors to test our three factors and interactions therewith. We tested the effects of our main plot factor—watering treatment—using its interaction with block. Subplot factors (usually species, sometimes timing) and their interaction with watering treatment were tested with an interaction term including block, watering treatment, and the factor being tested. Split-split plot factors and interactions therewith were tested with the full four-way interaction between block, watering treatment, species, and collection time when degrees of freedom were available. In cases when we were limited in the number of degrees of freedom, we removed all factor interactions with block from the model.

Table 1. Results from generalized linear mixed effects models of the frequency of hybridization (0/1), abundance of hybrid offspring, life-history, phenology and pollinator visitation patterns in wild and crop radish (Species) grown under four watering treatments (Watering Treatment), with phenological overlap (Timing) sampled across the growing season (Date) performed using SAS PROC GLIMMIX.

| Response and parameter | df  | F    | P     |
|------------------------|-----|------|-------|
| Hybridization events   |     |      |       |
| (Binary distribution,  |     |      |       |
| logit link function,  |     |      |       |
| n = 99                 |     |      |       |
| Watering Treatment     | 3,22| 0.73 | 0.55  |
| Timing                 | 2,57| 1.49 | 0.23  |
| Watering Treatment x Timing | 6,57| 0.55 | 0.77  |
| Number of hybrid offspring |     |      |       |
| (Lognormal distribution, identity link function, n = 47) | | |
| Watering Treatment     | 3,12| 0.42 | 0.74  |
| Timing                 | 2,12| 4.59 | 0.03  |
| Watering Treatment x Timing | 6,12| 0.37 | 0.89  |
| Days to flowering      |     |      |       |
| (Poisson distribution, log link function, n = 67) | | |
| Watering Treatment     | 3,24| 0.95 | 0.43  |
| Species                | 1,26| 202.08 | <0.0001 |
| Watering Treatment x Species | 3,26| 1.14 | 0.35  |
| Stem diameter          |     |      |       |
| (Gaussian distribution, identity link function, n = 68) | | |
| Watering Treatment     | 3,24| 0.58 | 0.64  |
| Species                | 1,27| 204.94 | <0.0001 |
| Species x Watering Treatment | 3,27| 0.33 | 0.80  |
| Overall abundance of flowers |     |      |       |
| (Lognormal distribution, n = 546) | | |
| Watering Treatment     | 3,24| 0.17 | 0.9135 |
| Species                | 1,31| 19.73 | 0.0001 |
| Watering Treatment x Species | 3,31| 0.21 | 0.8893 |
| Date                   | 10,400| 15.33 | <0.0001 |
| Date x Watering Treatment | 30,400| 1.03 | 0.4240 |
| Species x Date         | 9,400| 6.38 | <0.0001 |
| Species x Date x Watering Treatment | 26,400| 0.77 | 0.7842 |
| Relative flowering intensity |     |      |       |
| (Gaussian distribution, identity link function, n = 105) | | |
| Watering Treatment     | 3,23| 0.23 | 0.88  |
| Timing                 | 2,62| 9.86 | 0.0002 |
| Watering Treatment x Timing | 6,62| 1.24 | 0.30  |
| Pollinator movement    |     |      |       |
| (Lognormal distribution, identity link function, n = 19) | | |
| Watering Treatment     | 3,7 | 3.08 | 0.10  |

For the pollinator movement analysis, we lacked degrees of freedom to test the watering treatment x block interaction. For each response, underlying distribution, link function and sample size (n) are given. Significant effects are noted in bold.

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Next, we tested the degree to which the frequency of hybrid offspring produced relative to the total number of offspring produced by *R. raphanistrum* was related to soil moisture, relative flowering intensity and/or the relative frequency of insect movement between parental species. To do so, we used PROC GLIMMIX to fit a multiple regression model. Here we expected that plots with higher frequencies of switching by insects, under wetter conditions, and with lower RFI would exhibit the highest hybridization rates and thus produce the most hybrid offspring.

**Results**

**What is the Hybridization Rate between Crops and their Wild Relative?**

Overall, hybridization was rare across all plots; of the 8110 progeny genotyped, only 114 total hybrids were produced (3.4% hybridization rate) by 55 maternal plants (of 279 surviving maternal plants assessed, which corresponded to 86% of originally planted *R. raphanistrum* seedlings). When hybridization occurred, the frequency of hybrid offspring produced could be predicted by the date on which seeds were produced (Table 1, Fig 1). There were significantly more hybrid offspring produced late in the season rather than early in the season.

**Does Soil Moisture Affect Hybridization Rate by Altering Plant Size or Age at Reproduction, Floral Synchrony or Pollinator Movement between Parental Species?**

As expected, *R. sativus* plants started flowering later and at a larger size than *R. raphanistrum* (Fig 2, Table 1). As a result, wild radish plants produced an average of 45% more flowers than crop radish plants, though the precise difference in floral abundance changed with census date (Fig 2). The greatest difference was found on the second sampling date between July 15 and July 29, 2010 where wild radish had 22–67% more flowers. Watering treatment did not significantly affect the date of first flowering, stem diameter at first flowering, overall abundance of flowers, or the relative flowering intensity of parental populations. Early in the season, there were more *R. raphanistrum* flowers than *R. sativus* flowers (as measured by RFI) whereas by the end of the season, the number of open *R. sativus* and *R. raphanistrum* flowers were roughly equivalent (Table 1, Fig 3).

Across the season, we observed an average of 1 movement per 15 minute observation bout where insects switched from *R. raphanistrum* flowers to *R. sativus* flowers (i.e., movements that could have resulted in crop-wild hybridization). The frequency of movements that could result in pollen movement from *R. raphanistrum* to *R. sativus* plants did not differ significantly across watering treatments (Table 1, S1 Fig).

We expected positive relationships between hybridization and pollinator movement and soil moisture, and a negative relationship between hybridization and RFI. The frequency of hybrid offspring in the total seeds produced by *R. raphanistrum* increased as RFI decreased (i.e., as *R. sativus* flowers became relatively more abundant, $\beta = -0.041 \pm 0.016$, $n = 34$, $F_{1,22} = 6.75$, $P = 0.016$), but was not significantly related to insect movement ($P = 0.55$) or local soil moisture ($P = 0.53$, Table 2). Thus, RFI had the strongest influence on hybridization rate in this system.

**Discussion**

Despite their sexual compatibility, these results suggest that inter-specific hybridization rates between crop and wild radish are spatially variable and sensitive to the relative abundance of open flowers of each parental species (RFI). For instance, the production of hybrid offspring
occurred more frequently late in the reproductive season, or when the flowering intensity of crop and wild radish was approximately equal (equal numbers of open flowers for both species). Conversely, the production of hybrid offspring was less frequent when *R. raphanistrum* flowers were far more abundant than *R. sativus*, which occurred earlier in the reproductive season.

Contrary to our hypothesis, traits expected to be environmentally sensitive and thereby influence hybridization rates (e.g., plant size, RFI, overall floral abundance) did not respond to experimental manipulation of soil moisture. These results suggest that the timing of fruit

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**Fig 1.** Least squares means (± 95% CI) of the frequency of *Raphanus raphanistrum* x *R. sativus* hybrid progeny produced as predicted by the date of fruit harvest (early-, mid- or late-season). Overall means for each treatment are represented by black bars.

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harvest and its relationship to phenological overlap between parental species may be a stronger force in the formation of hybrid zones for *Raphanus* spp. than soil moisture or pollen vector movements. While this study suggests that species-specific temporal variation in relative flowering overlap predicts hybrid formation, it remains unclear how or if changes in the abiotic environment influence gene flow from crop-to-wild plant populations. Therefore, the consequences of anthropogenic climate change on evolutionary processes that maintain biological diversity, such as hybridization, remain unclear [58].

**Fig 2.** Least squares mean number of open flowers per harvest date (± 95% CI) across the flowering season of wild and crop radish, *Raphanus raphanistrum* and *R. sativus*, respectively. Early (E), Middle (M), and Late (L) sampling events are noted with arrows on the graph where developing fruits were tagged.

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Spatially variable and difficult-to-predict rates of interspecific hybridization is a particularly important observation, since hybridization can occur within a single generation and has important evolutionary consequences [59, 60]. Several studies have documented changes in hybridization rates in response to natural environmental gradients in a diversity of organisms [34, 61–66]. Yet, to our knowledge, this is the first published description of a controlled,
experimental manipulation of abiotic environmental variation on a population that subsequently explored the consequences for hybridization rates. This work reveals that hybridization rates can be difficult to predict based on manipulated abiotic conditions alone. However, once hybrids are formed, several studies have shown that their relative fitness is altered by variation in climatic conditions [35, 54, 67]. Experimental results presented in Campbell and Wendlandt [35] suggest that environmental conditions can play a significant role in realized hybridization rates in some species.

### Biotic Predictors of Hybridization

Hybridization rates typically respond to demographic differences in parental populations. On one hand, hybridization rates may be symmetric when parental populations are equally abundant. On the other hand, hybridization may be asymmetric when parental populations differ in abundance (e.g., [68–70]). Asymmetric hybridization events are thought to result from the relative abundance of male and female gametes from each parental population [56]. However, the evidence for the sensitivity of within-species hybridization rates to flowering effort is often incomplete and the causes are rarely experimentally attributed to differences in floral abundance between parental species. Here, we have documented that already low rates of hybridization (when parental populations produce similar numbers of flowers) decline further when one parental population produces far more flowers than the other. Despite many studies of hybridization between demographically asymmetric parental taxa (e.g., [68, 71, 72]), we are not aware of a study that has investigated the influence of relative floral abundance on patterns of hybridization. Floral production rates are known to vary through time and this variation appears to differ between crop and wild radish (and potentially between other crop-wild species complexes). Our results suggest predicting hybridization events may be more difficult across large geographic regions than previously recognized but may be most strongly influenced by the relative flowering intensity of both parental populations.

Pollinators often discriminate among flowers within and between species based on floral morphology, which can be highly sensitive to both abiotic and biotic variation (R. sativus [73]; R. raphanistrum [32]). Since both parental radish species are self-incompatible, only foraging events where pollinators move between plants result in seed production (especially since radish species were spatially separated in sub-plots, making it unlikely that wind or contact pollination could have occurred). Similarly, hybridization can occur only when foraging pollinators move between species. Furthermore, in an earlier study we have documented that increased water availability increased corolla diameter and potentially floral attractiveness.
We hypothesized that pollinator movements would correlate with actual hybridization events and thus rates of hybridization. Perhaps visiting insects did not respond to the microclimates within plots and thus did not influence gene flow.

**Introgression of Crop Genes into R. raphanistrum**

Many factors could influence rates of hybridization between cultivated radish (R. sativus) and its wild, weedy relative (R. raphanistrum), but generally, risk assessments have only considered physical and phenological separation, as well as genetic compatibilities [28, 47, 74]. For instance, hybridization rates between cultivated and wild R. sativus have been shown to range from 14–100% and generally decreased with increasing distance, exhibiting a typical leptokurtic distribution [28]. Surprisingly, despite adjacent planting locations, our crop and wild plants exhibited strikingly lower rates of hybridization than previously reported [28]. However, long-term studies monitoring the frequency of crop alleles in advanced-generation hybrid populations would be necessary to determine whether crop alleles could persist in wild populations over longer periods of time, even in the face of challenging environmental conditions (e.g., [74]). Variation in environmental conditions could create opportunities for periodic introgression from crop plants and maintain polymorphisms within weedy populations.

**Supporting Information**

**S1 Table.** The number of F₁ progeny genotyped based on flower colour for each plot and the number of mothers that contributed progeny to be genotyped per plot (maximum 9) during at least one collection point (Early, Mid or Late). The maximum number of progeny that could have been sampled per plot was: 540 seeds per plot. Progeny may not have been genotyped if the maternal plant did not flower on the sampling date, too few seeds per sample were produced, or the seed did not germinate or survive to flower before frost.

**S1 Fig.** The frequency of insect movements from crop to wild radish (Raphanus sativus and R. raphanistrum, respectively) across watering treatments (NR = No Rain, CU = Control Unsheltered, CS = Control Sheltered, DR = Double Rain) for nine plots per treatment (grey dots). Least squares mean values (±SD) across plots within a watering treatment are represented by black dots.

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References

1. IPCC, Intergovernmental Panel on Climate Change. Managing the risks of extreme events and disasters to advance climate change adaptation. Cambridge University Press, Cambridge, United Kingdom; 2012.

2. Angert AL, Crozier LG, Rissler LJ, Gilman SE, Tewksbury JJ, Chunco AJ. Do species’ traits predict recent shifts at expanding range edges? Ecol Lett. 2011; 14: 677–689. doi: 10.1111/j.1461-0248.2011.01620.x PMID: 21535340

3. Chen IC, Hill JK, Ohlemüller R, Roy DB, Thomas CD. Rapid range shifts of species associated with high levels of climate warming. Science. 2011; 333: 1024–1026. doi: 10.1126/science.1206432 PMID: 21852500

4. Hoffman AA, Sgro CM. Climate change and evolutionary adaptation. Nature. 2011; 470: 479–485. doi: 10.1038/nature09670 PMID: 21350480

5. Clegg MT. Measuring plant mating systems. Bioscience. 1980; 30: 814–818.

6. Sedlacek J, Schmid B, Matthies D, Albrecht M. Inbreeding depression under drought stress in the rare endemic Echium wildpretii (Boraginaceae) on Tenerife, Canary Islands. PLoS One. 2012. 7: e47415. doi: 10.1371/journal.pone.0047415 PMID: 23115645

7. Shiogg K, Pasinelli G, Walters JR, Daniels SJ. Inbreeding and experience affect response to climate change by endangered woodpeckers. P Roy Soc Lond B Bio. 2002; 269: 1153–1159.

8. Alsos IG, Ehrich D, Thuiller W, Eidesen PB, Trisch B, Schönswetter P, et al. Genetic consequences of climate change for northern plants. P Roy Soc B-Biol Sci. 2012; 279: 2042–2051.

9. Duminil J, Brown RP, Ewédjé EBK, Mardulyn P, Douct J, Hardy OJ. Large-scale pattern of genetic differentiation within African rainforest trees: insights on the roles of ecological gradients and past climate changes on the evolution of Erythrophleum spp. (Fabaceae). BMC Evol Biol. 2013; 13: 195. doi: 10.1186/1471-2148-13-195 PMID: 24028582

10. Zhu K, Woodall CW, Clark JS. Failure to migrate: lack of tree range expansion in response to climate change. Global Change Biol. 2012; 18: 1042–1052.

11. Hamrick JL, Godt MJW. Allozyme diversity in cultivated crops. Crop Sci. 1997; 37: 26–30.

12. Seehausen O. Conservation: losing biodiversity by reverse speciation. Curr Biol. 2006; 16: R334–R337. doi: 10.1016/j.cub.2006.03.080 PMID: 16682344

13. Bates BC, Kundzewicz ZW, Wu S, Palutikof JP. Climate change and water. Technical paper of the intergovernmental panel on climate change. IPCC Secretariat, Geneva; 2008.

14. Halpin PN. Global climate change and natural-area protection: management responses and research direction. Ecol Appl. 1997; 7: 828–843.

15. Walther GR, Post E, Convey P, Menzel A, Parmesan C, Beebee TJC, et al. Ecological responses to recent climate change. Nature. 2002; 416: 389–395. doi: 10.1038/416389a PMID: 11919621

16. Meehl GA, Arblaster JM, Tebaldi C. Contributions of natural and anthropogenic forcing to changes in temperature extremes over the United States. Geophys Res Lett. 2007; 34: L19709.

17. Meehl GA, Teng H. Multi-model changes in El Niño teleconnections over North America in a future warmer climate. Clim Dynam. 2007; 29: 779–790.

18. Matthews HD, Gillett NP, Stott PA, Zickfeld K. The proportionality of global warming to cumulative carbon emissions. Nature. 2009; 459: 829–833. doi: 10.1038/nature08047 PMID: 19516338
19. Parmesan C, Yohe G. A globally coherent fingerprint of climate change impacts across natural systems. Nature 2003; 421: 37–42. doi: 10.1038/nature01286 PMID: 12511946
20. Franks SJ, Sim S, Weis AE. Rapid evolution of flowering time by an annual plant in response to a climate fluctuation. P Natl Acad Sci USA. 2007; 104: 1278–1282.
21. Vigouroux Y, Mariac C, De Mita S, Pham J.-L., Gérad B, Kapran I, et al. Selection for earlier flowering crop associated with climatic variations in the Sahel. PloS ONE. 2011; 6: e19563. doi: 10.1371/journal.pone.0019563 PMID: 21573243
22. Ma X, Sukiran NL, Ma H, Su Z. Moderate drought causes dramatic floral transcriptomic reprogramming to ensure successful reproductive development in Arabidopsis. BMC Plant Biol. 2014; 14: 164. doi: 10.1186/1471-2229-14-164 PMID: 24928551
23. Schwarzbach AE, Donavan LA, Rieseberg LH. Transgressive character expression in a hybrid sunflower species. Am J Bot. 2001; 88: 270–277. PMID: 11222249
24. Arnold ML, Martin NH. Hybrid fitness across time and habitats. Trends Ecol Evol. 2010; 25: 530–536. doi: 10.1016/j.tree.2010.06.005 PMID: 20598770
25. Whitney KD, Randell RA, Rieseberg LH. Adaptive introgression of herbivore resistance traits in the weedy sunflower, Helianthus annuus. Am Nat. 2006; 167: 794–807. doi: 10.1086/504606 PMID: 16649157
26. Ellstrand NC, Meirmans P, Rong J, Bartsch D, Ghosh A, de Jong TJ, et al. Introduction of crop alleles into wild or weedy populations. Annu Rev Ecol Syst. 2014; 44: 325–345.
27. Barton NH, Hewitt GM. Analysis of hybrid zones. Annu Rev Ecol Syst. 1985; 16: 113–148. Stable URL: http://www.jstor.org/stable/2097045
28. Klinger T, Elam DR, Ellstrand NC. Radish as a model system for the study of engineered gene escape rates via crop-weed mating. Conserv Biol. 1991; 5: 531–535.
29. Field DL, Ayre DJ, Whelan RJ, Young AG. The importance of pre-mating barriers and the local demographic context for contemporary mating patterns in hybrid zones of Eucalyptus aggregata and Eucalyptus rubida. Mol Ecol. 2011; 20: 2367–2379. doi: 10.1111/j.1365-294X.2011.05054.x PMID: 21375638
30. Snow AA, Andow DA, Gepts P, Hallerman EM, Power A, Tiedje JM, et al. Genetically engineered organisms and the environment: current status and recommendations. Ecol Appl. 2005; 15: 377–404.
31. Cummings CL, Alexander HM. Population ecology of wild sunflowers: effects of seed density and post-dispersal vertebrate seed predators. Oecologia. 2002; 120: 274–280.
32. Pertl M, Hauser TP, Damgaard C, Jørgensen RB. Male fitness of oilseed rape (Brassica napus), weedy B. rapa and their F1 hybrids when pollinating B. rapa seeds. Heredity. 2002; 89: 212–218. doi: 10.1038/sj.hdy.6800131 PMID: 12209392
33. Marques I, Rosselló-Graell A, Draper D, Iriondo JM. Pollination patterns limit hybridization between two sympatric species of Narcissus (Amaryllidaceae). Am J Bot. 2007; 94: 1352–1359. doi: 10.3732/ajb.94.8.1352 PMID: 21636503
34. Garroway CJ, Bowman J, Cascaden TJ, Holloway GL, Mahan CG, Malcolm JR, et al. Climate change induced hybridization in flying squirrels. Global Change Biol. 2010; 16: 113–121.
35. Campbell DR, Wendlandt C. Altered precipitation affects plant hybrids differently than their parental species. Am J Bot. 2013; 100: 1322–1331. doi: 10.3732/ajb.1200473 PMID: 23748678
36. Bolmgren K, Lörenberg K. Herbarium data reveal an association between fleshy fruit type and earlier flowering time. Int J Plant Sci. 2005; 166: 663–670.
37. Forrest J, Inouye DW, Thomson JD. Flowering phenology in subalpine meadows: Does climate variation influence community co-flowering patterns? Ecology. 2010; 91: 431–440. PMID: 20392008
38. Farooq M, Wahid A, Kobayashi N, Fujita D, Basra SMA. Plant drought stress: effects, mechanisms and management. Agron Sustain Dev. 2009; 29: 185–212.
39. Anjum SA, Xie XY, Wang LC, Saleem MF, Man C, Lei W. Morphological, physiological and biochemical responses of plants to drought stress. Afr J Agric Res. 2011; 6: 2026–2032.
40. Eckhart VM. The effects of floral display on pollinator visitation vary among populations of Phacelia lineariis (Hydrophyllaceae). Evol Ecol. 1991; 5: 370–384.
41. Cruzan MB, Hamrick JL, Arnold ML, Bennett BD. Mating system variation in hybridizing irises: effects of phenology and floral densities on family outcrossing rates. Heredity 1994; 72: 95–105.
42. Brunet J, Sweet HR. Impact of insect pollinator group and floral display size on outcrossing rate. Evolution. 2006; 60: 234–246. PMID: 16610316
43. Kennedy BF, Elle E. The reproductive assurance benefit of selfing: importance of flower size and population size. Oecologia. 2008; 155: 469–477. doi: 10.1007/s00442-007-0924-7 PMID: 18066603
44. Conner JK, Rush S, Jennetten P. Measurements of natural selection on floral traits in wild radish (Raphanus raphanistrum). 1. Selection through lifetime female fitness. Evolution. 1996; 50: 1127–1136.

45. Campbell LG, Parker RJM, Blakelock G, Pirimova N, Mercer KL. Maternal environment influences propagule pressure of an invasive plant, Raphanus raphanistrum (Brassicaceae). Int J Plant Sci. 2015; 176: 393–403.

46. Pirimova N., Parker A. J., and Campbell L. G.. Does altering local water availability for an invasive plant (Raphanus raphanistrum) affect floral morphology and reproductive potential? Am J Undergrad Res. 2015; 12: 63–72.

47. Snow AA, Uthus KL, Culley TM. Fitness of hybrids between weedy and cultivated radish: Implications for weed evolution. Ecol Appl. 2001; 11: 934–943.

48. Hegde SG, Nason JD, Clegg JM, Ellstrand NC. The evolution of California’s wild radish has resulted in the extinction of its progenitors. Evolution. 2006; 60:1187–1197. PMID: 16892969

49. Gomez C, Despinoy M, Hamon S, Hamon P, Salmon D, Akaffou DS, et al. Shift in precipitation regime promotes interspecific hybridization of introduced Coffea species. Ecol Evol. 2016

50. Panetos CA, Baker HG. The origin of variation in wild Raphanus sativus (Cruciferae) in California. Genetica. 1967; 38: 243–274.

51. Campbell LG, Snow AA, Sweeney PM. When divergent life histories hybridize: insights into adaptive life-history traits in an annual weed. New Phytol. 2009; 184: 806–818. doi: 10.1111/j.1469-8137.2009.03036.x PMID: 19814778

52. Lee TN, Snow AA. Pollinator preferences and the persistence of crop genes in wild radish populations (Raphanus raphanistrum, Brassicaceae). Am J Bot. 1998; 85:333–339. PMID: 21684916

53. Snow AA, Campbell LG. Can feral radishes become weeds? In: Gressel J, ed. Crop Ferality and Volunteering. CRC Press; 2005. Pp. 193–208.

54. Campbell LG, Snow AA, Ridley CE. Weed evolution after crop gene introgression: greater survival and fecundity of hybrids in a new environment. Ecol Lett. 2006; 9: 1198–1209. doi: 10.1111/j.1461-0248.2006.00974.x PMID: 17040322

55. Conner JK, Via S. Patterns of phenotypic and genetic correlations among morphological and life-history traits in wild radish, Raphanus raphanistrum, Evolution. 1993; 47:704–711.

56. Rhymer JM, Simberloff D. Extinction by hybridization and introgression. Annu Rev Ecol Syst. 1996; 27: 83–109.

57. Borror DJ, Delong DM, Triplehorn CA. An introduction to the study of insects. Saunders Press; 1981.

58. Myers N, Knoll AH. The biotic crisis and the future of evolution. P Natl Acad Sci USA. 2001; 98: 5389–5392.

59. Seehausen O. Hybridization and adaptive radiation. Trends Ecol Evol. 2004; 19: 198–207. doi: 10.1016/j.tree.2004.01.003 PMID: 16701254

60. Whitney KD, Ahern JR, Campbell LG, Albert LP, King MS. Patterns of hybridization in plants. Perspect Plant Ecol. 2010; 12: 175–182.

61. Chuncos AJ. Hybridization in a warmer world. Ecol Evol. 2014; 4: 2019–2031. doi: 10.1002/ece3.1052 PMID: 24963394

62. Mimura M, Mishima M, Lascoux M, Yahara T. Range shift and introgression of the rear and leading populations in two ecologically distinct Rubus species. BMC Evol Biol. 2014; 14: 209.

63. Muhlfeld CC, Kovach RP, Jones LA, Al-Chokhachy R, Boyer MC, Leary RF, et al. Invasive hybridization in a threatened species is accelerated by climate change. Nat Clim Chang. 2014; 4: 620–624.

64. Sánchez-Guillén RA, Córdoba-Aguilar A, Hansson B, Oll J, Wellenreuther M. Evolutionary consequences of climate-induced range shifts in insects. Biol Rev. 2015.

65. de La Torre A, Ingvarsson PK, Aitken SN. Genetic architecture and genomic patterns of gene flow between hybridizing species of Picea. Heredity. 2015; 115: 153–164. doi: 10.1038/hdy.2015.19 PMID: 25806545

66. Li Y, Maki M. Variation in the frequency and extent of hybridization between Leucosceptrum japonicum and L. stellipilum (Lamiales) in the central Japanese mainland. PloS ONE. 2015; 10: e0116411. doi: 10.1371/journal.pone.0116411 PMID: 25738505

67. Teitel Z, Laursen AE, Campbell LG. Germination rates of weedy radish populations (Raphanus spp.) altered by crop-wild hybridisation, not human-mediated changes to soil moisture. Weed Res. 2016; 56: 149–158.

68. Burgess KS, Morgan M, DeVerno LL, Husband BC. Asymmetrical introgression between two Morus species (M. alba, M. rubra) that differ in abundance. Mol Ecol. 2005; 14: 3471–3483 doi: 10.1111/j.1365-294X.2005.02670.x PMID: 16156816
69. Prentis P, White E, Radford IJ, Lowe AJ, Clarke AR. Can hybridization cause local extinction: the case for demographic swamping of the Australian native, Senecio pinnatifolius, by the invasive, S. madagascariensis? New Phytol. 2007; 176: 902-912 doi: 10.1111/j.1469-8137.2007.02217.x PMID: 17850249

70. Field DL, Ayre DJ, Whelan RJ, Young AG. Relative frequency of sympatric species influences rates of interspecific hybridization, seed production and seedling performance in the uncommon Eucalyptus aggregata. J Ecol. 2008; 96: 1198–1210

71. Brochmann C. Hybridization and distribution of Argyranthemum coronopifolium (Asteraceae-Anthemideae) in the Canary Islands. Nord J Bot. 1984; 4: 729–736.

72. Bleeker W, Hurka H. Introgressive hybridization in Rorippa (Brassicaceae): gene flow and its consequences in natural and anthropogenic habitats. Mol Ecol. 2001; 10: 2013–2022. PMID: 11555244

73. Stanton M. Reproductive biology of petal color variants in wild populations of Raphanus sativus. 1. Pollinator response to color morphs. Am J Bot. 1987; 74: 178–187.

74. Snow AA, Culley TM, Campbell LG, Hegde SG, Ellstrand NC. Long-term persistence of crop alleles in weed populations. New Phytol. 2010; 186: 537–548. doi: 10.1111/j.1469-8137.2009.03172.x PMID: 20122132