RICE UNIVERSITY

Evolutionary responses to global change: an experimental test of the effect of altered precipitation on hybridization rates in Sunflower *(Helianthus)*

by

Michelle Sneck

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Master of Arts

APPROVED, THESIS COMMITTEE

Dr. Kenneth Whitney, Chair
Associate Professor

Dr. Tom Miller
Assistant Professor

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HOUSTON, TEXAS
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ABSTRACT

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Climate change is rapidly altering natural ecosystems. Plastic and adaptive responses to climate change (i.e., range shifts and phenology) have been widely noted across taxa. However, the effects of climate change on evolutionary processes such as interspecific gene flow (hybridization) are less well known. In this study, we quantified hybridization rates in response to experimental manipulations of rainfall, an important dimension of global change. We used rain-out shelters in the field and quantified rates of hybridization between two congeners, *Helianthus annuus* (common sunflower) and *H. petiolaris* (prairie sunflower). We found that *H. annuus* maternal plants produced more hybrid progeny than *H. petiolaris* maternal plants, with a trend for decreased rates of hybridization with increased soil moisture (when rain-out shelters were absent). Furthermore, the relative number of open inflorescences of each species predicted hybridization rates. Thus, this study demonstrates how changing environmental conditions, specifically precipitation, could influence hybridization rates.

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Contents

Acknowledgments iii
Contents iv
List of Figures v
List of Tables i
References 23

List of Figures

Fig. 1. Diagram of half of the plot layout. P symbolizes *H. petiolaris* individuals and A symbolizes *H. annuus* individuals. Numerical subscripts indicate individual plants. 33

Fig. 2. Rain-out shelters used to capture rainfall. Rainfall hit the transparent roofing, was caught in the rain gutter, and drained into a rain barrel. The rain was then redistributed using a 4-hp water pump and tractor. 34

Fig. 3. The fraction of F_1 *H. annuus* individuals confirmed to be of hybrid status as a function of leaf abaxial trichome density. No individuals possessing >50 abaxial trichomes/mm^2 were hybrid. 35

Fig. 4. The external transcribed space region of the 18s rDNA (ETS) visualized using gel electrophoresis. Hybrids (H) possess two bands while each parent expresses a single unique band (P, *H. petiolaris*; A, *H. annuus*). 36

Fig. 5. Average soil moisture across the season for each rainfall manipulation treatment. 37

Fig 6. Average hybridization rate (fraction of progeny that were hybrids) for each species across four rainfall treatments. 38

Fig. 7. The relationship between soil moisture and average hybridization rate of *H. annuus* plants in plots with (n = 27) and without (n = 9) rainout shelters. 39

Fig 8. Hybridization rates of *H. annuus* (A) and *H. petiolaris* (B) predicted by relative flowering intensity (RFI). Each symbol represents a single plot on one of four sampling dates. Plot 16 was removed from the analysis due to complete *H. peitolaris* mortality in this plot. In B, one treatment out of four was plotted to demonstrate the slope of the curve, but for simplicity the other three treatments are not shown. 40

List of Tables

Table 1. Linear model analyzing the effect of average soil moisture and shelter presence/absence on *H. annuus* hybridization rate. 41

INTRODUCTION

Increased concentrations of atmospheric CO_2 have resulted in a 0.2^{o}C mean increase in global surface temperatures each decade over the past 30 years (Hansen 2006). Along with future temperature shifts, current climate models also predict an increase in the frequency of extreme precipitation fluctuations, including flooding and drought throughout the globe (Allan and Soden 2008, Min et al. 2011). Understanding and predicting the consequences of climate change for natural populations is of critical importance.

Over the past few decades, many natural populations across taxonomic groups have responded to climate change (reviewed in Parmesan 2006). A widely observed response is the movement of species ranges as organisms attempt to track optimal environmental conditions. For example, populations of butterflies (Boggs and Murphy 1997, Parmesan 2006), plants (Kelly and Goulden 2008), and mammals (Garroway et al. 2010) have shifted northward, a pattern often attributed to an increase in mean temperatures towards the lower latitudinal extent of their ranges. The most frequently recorded responses are changes in the expression of life history traits, such as the timing of emergence or reproduction (phenology), which are often cued by abiotic factors (Parmesan 2006). Phenological changes have been pervasive in flowering plants, with many species flowering earlier in the season, which is presumed to be initiated in part by earlier snow melt due to warmer average winter temperatures (Saino et al. 2009, Franks and Weis 2009, Rousi et al. 2011, Galloway and Burgess 2012).

Ecological responses to climate change that are plastic or entail the movement of populations across the landscape have been consistently recorded over time, are easily observed, and act as important components to predictions of future population distributions and abundances. However, it has recently become clear that climate change may have equally important effects on evolutionary processes including natural selection, genetic drift, and gene flow – all of which can influence the demographic and evolutionary trajectories of populations and species (Merilä 2012, Anderson et al. 2012a). Unfortunately, detecting the signal of natural selection in response to a changing climate has been challenging and as a result, such studies largely lag behind investigations of ecological responses (Gienapp et al. 2008, Merilä 2012). This is partly explained by the dearth of genetic information associated with many putatively adaptive traits. However, select studies have revealed changes in allele frequencies due to selective forces associated with climate change, especially in genes controlling expressions of thermal tolerance (Bradshaw 1991, James et al. 2002, Anderson et al. 2012b). An additional reason for the lack of evidence for evolution by natural selection in response to climate change could be low amounts of heritable genetic variation for adaptive traits (Kirkpatrick and Barton 1997, Merilä 2012). In addition to altering patterns of natural selection, climate change may impact genetic drift. Available genetic variation may be reduced by the predicted decline in the area of ideal habitats in the future, resulting in overall smaller population sizes (Travis 2003, Keith et al. 2008). These population contractions may have severe consequences for species long-term persistence, because diminished, genetically poor populations are more prone to genetic drift and inbreeding depression (increased homozygosity) (Willi et al. 2007). In fact, genetic drift resulting in

a loss of genetic variation has already been observed as a consequence of climate change and in some cases has caused population extirpations (Stockwell et al. 2003, Morrison and Hik 2007, Angeloni et al. 2011, Bijlsma and Loeschcke 2011).

As organisms move throughout the landscape in response to climate change, populations or taxa undergoing range shifts may collide, reducing prezygotic reproductive barriers between them. This could not only increase gene flow between locally adapted populations, but also between genetically and ecologically differentiated species (hybridization) (Berteaux et al. 2004, Franks and Weis 2009, Crispo et al. 2011, Paul et al. 2011, Arenas et al. 2012). In addition to species range shifts, other commonly observed organismal responses to climate change could influence patterns of interspecific hybridization (Beatty et al. 2010, Hoffmann and Sgrò 2011, Garroway et al. 2010). For instance, phenological responses to climate change may also alter gene flow between species (Heard et al. 2011) because they present the opportunity for either increased or decreased reproductive synchrony over time (Munguía-Rosas et al. 2011). Specifically in flowering plants, recent studies have reported changes in relative flowering overlap between species and predict that historic patterns of gene flow may be altered under future climate regimes (Miller-Rushing et al. 2007, Forrest and Thomson 2011, Dunnell and Travers 2011). Additionally, observed climate-induced changes in pollinator abundance and behavior may influence interspecific pollen transfer (Hegland et al. 2009, Forrest and Thomson 2011) as the rate of pollinator movements between species has been experimentally correlated with rates of hybridization (Campbell et al. 2002).

Investigations of the effect of climate change on interspecific hybridization have so far been limited to one animal and one plant system. First, in an observational study, Garroway et al. (2010) found evidence of a recently created hybrid zone between two flying squirrel species, *Glaucomys sabrinus* and *G. volans,* whose ranges have converged over the past decade. While this study is the first to document hybridization in the context of putatively climate-induced range shifts, it is observational and therefore cannot causally connect climate change to rates of interspecific gene flow. Second, in an experimental study, Campbell and Wendlandt (*in review*) revealed that natural hybrids of the alpine herbs *Ipomopsis tenuituba* and *I. aggregata* have higher fitness relative to both parental species when soil moisture is reduced, while retaining similar fitness at high levels of soil moisture. These results suggest that hybrids may have a selective advantage over their parents if future climate regimes result in a drier climate in the alpine habitats where these plants grow. This study focused on the performance of existing hybrids but did not examine rates of hybrid formation. An understanding of both processes is needed to predict the effects of climate change on interspecific gene flow. Clearly, experimental studies are needed that examine rates of both hybrid formation and persistence.

In this study, we experimentally manipulated rainfall with the use of rain-out shelters and quantified hybridization rates between two congeners known to hybridize in nature, *Helianthus annuus* (common sunflower) and *H. petiolaris* (prairie sunflower). The experiment was designed to mimic projections of future rainfall patterns in which droughts and flooding are predicted (Min et al. 2011) by creating both dry (no rainfall from July 17th – Sept 29th) and wet (twice the amount of ambient rainfall) conditions in the field; these treatments were compared to a treatment representing ambient conditions

(natural rainfall). Specifically, we asked two questions: 1) Does precipitation affect hybridization rate (the proportion of progeny that are hybrid)? And, 2) Can hybridization rate be explained by the relative flowering intensity of the two parental species (i.e., does phenological overlap predict hybridization)? To our knowledge, this is the first study to experimentally examine the effects of simulated climatic shifts on rates of interspecific hybrid formation.

METHODS

Study Species --- *Helianthus annuus* (common sunflower) and *H. petiolaris* (prairie sunflower) (Family: Asteracae) are both annual plants native to the central and western United States. The species are ecologically and morphologically distinct (Heiser 1961) and can be commonly found occupying disturbed grasslands throughout their widespread and largely overlapping ranges (Rogers et al. 1982). As self-incompatible plants, *H. annuus* and *H. petiolaris* rely on insect pollinators for reproduction and have been documented to readily hybridize in nature despite some prezygotic (Rieseberg et al. 1995, 1998) and post-zygotic (Lai 2005) reproductive barriers. Past hybridization events between *H. annuus* and *H. petiolaris* have resulted in three novel homoploid hybrid species*: H. anomalus*, *H deserticola,* and *H. paradoxus* (Rieseberg et al. 2003).

Study Location --- Our experiment was performed on the Waterman Farm and Turf Grass Experimental Station of the Ohio State University in Columbus, OH, USA (40°80’ N latitude and 83°01’ W longitude) from July 6-October 11, 2010. This location is within the range of both *H. annuus* and *H. petiolaris* (Rogers et al. 1982). Average annual temperature is 11°C and precipitation is ≈932 mm per year (USDA-SCS, 1980).

Soil type varied across the farm with areas characterized as both Crosby silt loam or Aeric Ochraqualf (USDA classification) and Stagnic Luvisol (Food and Agriculture Organization classification).

Experimental Design --- To examine the influence of precipitation on hybridization rates between *H. annuus* and *H. petiolaris,* thirty-six plots containing both parental species were established and subject to experimental rainfall manipulations. At the beginning of the season, each plot (n=36) was sprayed with Roundup (Monsanto, St. Louis, MO) to kill the existing vegetation and was tilled prior to transplanting; subsequent weeds were removed manually throughout the season. To each plot we randomly assigned nine plants per species and transplanted them between June 6th -10th, 2010 for a total of 18 *Helianthus* plants per plot. To account for variation in abiotic conditions within the site, the 36 experimental plots were grouped into nine blocks, each consisting of four plots each assigned to a different rainfall manipulation treatment (described below). These blocks were located haphazardly throughout the 500-acre station. Each species occupied approximately one quarter of a 3.05 m x 2.44 m plot with the nine plants arranged in a three by three planting grid; individual plants were planted approximately 30 cm apart (Fig. 1). Species were planted in this pattern to reflect the spatial orientation of population boundaries and hybrid zones that naturally occur throughout their overlapping ranges. In the remaining area of each plot we planted nine individuals of each of two hybridizing radish species (*Raphanus sativus* and *R. raphanistrum*); results for these species will be presented elsewhere. Each plant received 0.5 L of water upon transplanting.

Plots within a block were randomly assigned to one of four levels of rainfall manipulation: control open, control sheltered, rainfall addition, and rainfall exclusion. To control and manipulate rainfall, rain-out shelters were placed above the control-sheltered, wet, and dry plots but not the control-open plots (Fig. 2). By including a control plot without a shelter, we can account for possible unintended effects of shelter presence upon hybridization rates. A shelter consisted of a wooden frame with a solid, transparent polycarbonate roof (Waldo and Associates, Toledo, OH), which was built at a slight angle to divert water from the plot into a 227-liter rain barrel. Impermeable sheets of greenhouse plastic (Waldo and Associates, Toledo, OH) 76 cm wide were placed on the ground surrounding each plot with a rain-out shelter to prevent nearby rainfall from diffusing into the plot, however they did not extend below the surface of the soil so rainfall seepage beneath the ground plastic was not prevented. The peripheral greenhouse plastic of the rain-out shelters in the control shelter and wet treatment plots were punctured to allow for immediate water absorption. Similar rain-out treatment designs have been used to simulate effects of climate change on natural systems (e.g., (Yahdjian and Sala 2002, Levine et al. 2010, Salamin et al. 2010)). Control-sheltered plots received all water captured in the plot's rain barrel. Plants in the dry treatment did not receive any captured rain-water after transplanting (although some rainfall may have blown in from the sides of the shelter). Wet plots received the rainwater captured in the rain barrels of both wet and dry plots, effectively doubling the amount of water received relative to the ambient levels experienced by both types of control plots. Water was relocated from barrels to plots within 48hrs of each rainfall event throughout the season.

Seed Sources and Plant Propagation --- The parental generation of *Helianthus annuus* achenes was initially collected on September 30, 2007 from a wild population from the Desoto Wildlife Refuge in western Iowa (41° 32.8' N 96° 2.1' W). From descendants of this initial population, 30 achenes were collected and germinated from each of 30 dams. Of these 900 achenes, 324 germinated and survived to the seedling stage. The parental generation of *Helianthus petiolaris* was propagated by the USDA and grown from achenes originally sourced from Illinois (PI 478307; Lot: 03ncai01; GRIN, USDA). A total of 1,000 *H. petiolaris* achenes were germinated but only 324 individuals were used in order to match the *H. annuus* count. In early May, 2010, all achenes were released from dormancy via nicking, and germinated on filter paper; after growing an extended radicle they were transplanted into peat pots (6 × 10 cm, Jiffy Products of America, Inc., Lorain, OH) containing soil-less peat mixture (ProMix BX, Premier Horticulture Ltd., Rivière-du-Loup, Canada). Seedlings were grown in a greenhouse for approximately 4 weeks before transplanting to the field.

Data Collection --- The numbers of open and senesced inflorescences of *H. annuus* and *H. petiolaris* individuals were counted twelve times (once per week) during the 2010 growing season between July 1 and October 11 to quantify flowering overlap. To retain achenes for use in the quantification of hybridization rates (below), between 2 and 6 mature (post-pollination), haphazardly chosen inflorescences with senesced disk flowers per plant were bagged and labeled throughout the season on four dates: early (August 19th), early-middle (August 26th), late-middle (September 1st), and late (October 1st). Bags were 11.5 cm x 10 cm and were made from DelStar plastic mesh

(DelStar Technologies, Delaware). Achenes were allowed to ripen and were collected at the end of the season.

Soil moisture was measured at the center of each plot approximately 8 hours after each watering treatment was applied (July 27 & 29, August 3, 7 & 11, September 2, 25, 29). These dates correspond to bouts of natural rainfall. For the first sampling period, a single soil moisture reading was taken from the center of each plot. For every subsequent sampling period, average soil moisture per plot was calculated from three separate readings taken from central locations within each plot. However, there were two days when rainfall treatments were applied after a rainfall, but soil moisture was not recorded (August 16, 23).

Progeny Sampling --- The frequency of hybrid progeny was determined in subsets of the achenes collected from maternal parents of both species from each plot. Three achenes from each of the six individual plants (three *H. annuus* and three *H. petiolaris*) from each plot at every bagging time period (August 19, August 26, September 1, October 1) totaling to 12 achenes per plant were germinated and grown in a greenhouse at Rice University in Houston, TX. This makes for an overall total of 72 progeny from each of the 36 plots. Maternal plants were selected to equally represent proximity to the congeneric species within each plot (i.e., the three *H. annuus* maternal plants selected were 30, 75 and 120 cm from the nearest *H. petiolaris* individual), to account for differences in the likelihood of receiving interspecific pollen.

Distinguishing Hybrids --- Putative hybrids were first identified via a screening procedure based on morphological characters (average abaxial and adaxial leaf glandular

trichome density) and then confirmed with the use of molecular techniques. One parental species (*H. annuus*) is characterized by numerous leaf glandular trichomes (Whitney et al. 2006), while the other (*H. petiolaris*) typically has glabrous or nearly glabrous leaves (Heiser 1961).

We expected hybrid progeny of *H. annuus* maternal parents would have low trichome counts compared to the high trichome counts found in pure *H. annuus* individuals (Heather Rowe, pers. comm.). Therefore, with the use of a diagnostic molecular marker discussed below, we genotyped all progeny with <30 abaxial trichomes/mm^2 (n=289) and a sampling of individuals with >=30 abaxial trichomes/ mm^2 (n=45), finding 82 and 4 hybrids in these two groups, respectively. As demonstrated by Fig. 3, the fraction of hybrid individuals decreases with increasing trichome counts and becomes very small (<3%) once the threshold of 30 abaxial trichomes/mm^2 is crossed. We then used discriminant analysis to classify ungenotyped individuals with >30 abaxial trichomes/mm^2 (n=860) into "putative hybrid" and "putative non-hybrid" classes using both abaxial and adaxial trichome counts (the training dataset was n=321 genotyped individuals). The resulting "putative hybrid" class (n=12) included individuals that were placed between 30-100% probability of hybrid status; these were then genotyped, and were only counted as hybrids if the genotyping confirmed actual hybrid status (n=3).

Based on results from *H. annuus* and from the experience of others (Heather Rowe, pers. comm.), we expected that hybrid progeny from *H. petiolaris* maternal plants would bear trichomes. We genotyped all trichome-bearing progeny in our sample (n=86) and found a total of nine hybrids. We then genotyped a large sample of trichome-free

progeny (n=60) and found no hybrids, leading us to classify all remaining trichome-free progeny (n=1,004) as non-hybrid.

Molecular Techniques for Assessing Hybrid Status --- DNA from putative hybrids was isolated from plant leaf tissue using DNeasy Tissue Kit (Qiagen Inc.). One diagnostic locus was used to assess hybrid status; the External Transcribed Spacer (ETS) region, ETS1f and the reverse primer 18s2L (TGACTACTGGCATCAACCAG), is known to be expressed uniquely in both parental taxa as single segments of differing lengths, while hybrids express both parental segments (Linder 2000) (Fig. 4). PCR occurred in a 25 μL volume with 20 ng of DNA; 1.5 mM of MgCl2; 0.8 mM dNTP; 1.5 mM BSA; 0.5 units of Taq polymerase; and 0.2 μM each of ETS1f and 18s2l primers (Linder 2000). Reactions were run on a thermocycler (Eppendorf Mastercycler Gradient Thermocycler) with the following program: 94^{o}C for 180 sec, followed by 30 cylces of 94^{o}C for 30 sec, 52^{o}C for 30 sec, and 72^{o}C for 90 sec, with a final step of 72^{o}C 5 min. Following amplification, 3 μL of reaction mixture for each specimen was run using electrophoresis on a 0.8% agarose gel in 1X TBE buffer at ~180 volts for 50 minutes to ensure full separation of the bands. Amplifications were visualized using SYBR® Safe (Invitrogen, USA) and confirmed with a standard 1-Kb ladder (Promega, USA). Bands between 0.8 and 2.5 kb confirmed successful amplification of the ETS region (Fig. 4). Pure *H. annuus* and *H. petiolaris* individuals acted as negative controls in both amplification and visualization of all specimens.

Statistical Analysis --- We first verified that the rainfall manipulation resulted in significantly different levels of average soil moisture in each treatment type (dry, control

shelter, control open, wet). A repeated measures mixed model was used to assess if average soil moisture differed between soil moisture treatments. We utilized an autoregressive covariance matrix that included the interaction between treatment type and date of moisture measurement. Block was treated as a random effect. *Post-hoc* comparisons among treatments were done using Tukey's HSD test.

We calculated an average hybridization rate for each maternal species in each plot. The number of progeny produced varied across the four sampling periods in any given plot, making it inappropriate to simply average our four estimates of hybridization rate. Instead, plot-level hybridization rates for each maternal species were calculated as

$$\sum_{i=1}^{4}\left(\frac{Hybrid\ progeny}{Total\ progeny}\times\frac{Open\ Inflorescences}{Total\ Inflorescences}\right)$$

We then examined determinants of hybridization rates using generalized linear and mixed models with PROC GLM, PROC MIXED, PROC LOGISTIC, and PROC REG in SAS version 9.3 (SAS Institute, Cary, NC).

Does precipitation affect hybridization rate? --- We addressed this question in two ways. First, we examined the effects of the rainfall treatments as a categorical variable on hybridization rate, which was arcsine square root transformed to achieve normality. The generalized linear mixed model included treatment (four levels) and maternal species (two levels) as independent variables along with their interaction term; block was included as a random variable. *Post-hoc* comparisons of hybridization rate among treatments were established using Tukey's HSD test.

Second, the average soil moisture associated with each plot across the season was used to explore the continuous relationship between soil moisture and hybridization rate. For *H. annuus* progeny, the generalized linear model included average plot soil moisture, shelter and their interaction as independent variables; shelter was a binary variable (1= shelter, 0= no shelter; shelters were present in all plots except for the control open treatment). *H. annuus* hybridization rate was again arcsine square root transformed to improve normality. For *H. petiolaris* progeny, because overall rates of hybridization were quite low (resulting in numerous zeroes), we analyzed plot-level hybridization as a binary variable (yes/no) via logistic regression. Independent variables were the same as in the *H. annuus* analysis above. An outlier in the soil moisture data set was identified using PROC REG in SAS version 9.3 and removed from all analyses (Cook's D = 0.4) (SAS institute, Cary, NC).

Can hybridization rate be predicted by the relative flowering intensity of the two parental species? --- To determine whether phenological data could be used to predict hybridization rates, we calculated the relative flowering intensity (RFI) in each sampling period as the ratio of open *H. annuus* inflorescences to open *H. petiolaris* inflorescences. Note that the hybridization rates examined here were specific to each sampling period (four rates per plot) and differed from the average plot-level rates (one rate per plot) analyzed in the previous section. A generalized linear model for *H. annuus* assessed whether RFI (square root transformed to normalize very large and small values), treatment, and the RFI*treatment interaction predicted hybridization rates; plot (nested within treatment) was included as a random term in the generalized linear model. A logistic regression was used to assess the relationship between the probability of

hybridization and RFI for *H. petiolaris*. The logistic model included only RFI and treatment (AIC=63.889) as predictor variables because the interaction between RFI and treatment did not improve model fit (AIC=69.456). A separate generalized linear model assessed whether RFI differed among treatments, and a simple regression investigated the relationship between RFI and average soil moisture.

RESULTS

Experimental treatments altered soil moisture --- Average soil moisture differed significantly among experimental treatments (treatment $F_{3,32} = 63.72$, $P < 0.0001$; date of moisture measurement $F_{7,222} = 20.76$, $P < 0.0001$, treatment*time interaction $F_{21,222} = 4.87$, $P < 0.0001$; Fig. 5). The random term block ($Z_7 = 1.24$, $P = 0.1075$) and the interaction between block and date ($Z_7 = 0.14$, $P = 0.444$) were not significant within the model. Soil moisture did not differ between the control open and control shelter plots (Tukey's HSD test, $t_{1,32} = 1.72$, $P = 0.3324$). In contrast, soil moisture differed significantly between the dry and wet plots ($t_{1,32} = -13.56$, $P < 0.0001$) as wet plots on average possessed 2.85 times more soil moisture than dry plots. Both dry and wet plots differed from the control open and control shelter plots (dry: control open $t_{1,32} = 9.07$, $P < 0.0001$, control shelter $t_{1,32} = 7.35$, $P < 0.0001$; wet: control open $t_{1,32} = -4.54$, $P = <0.001$, control shelter $t_{1,32} = -6.24$, $P < 0.0001$).

Does precipitation affect hybridization rate? --- There was no main effect of the rainfall treatment on hybridization rate ($F_{3,56} = 0.91$, $P = 0.4433$) but we found significant effects of maternal species ($F_{1,56} = 43.59$, $P = < 0.0001$) and a significant treatment*maternal species interaction ($F_{3,56} = 3.08$, $P = 0.0348$), indicating that the

rainfall treatments affected hybridization rates of *H. annuus* and *H. petiolaris* differently. Hybridization rates throughout the season were on average 18 times higher for *H. annuus* maternal plants than *H. petiolaris* maternal plants (Fig. 6). For *H. annuus*, rainfall treatments did not influence hybridization rate ($F_{3,24} = 2.27$, $P = 0.1065$); however, there was a trend for more hybridization in control shelter plots than in control open plots (Tukey's HSD test, $t_{1,24} = -2.50$, $P = 0.0695$). For *H. petiolaris*, rainfall treatments did not influence the hybridization rate ($F_{3,24} = 2.09$, $P = 0.1277$).

In additional analyses, the influence of average soil moisture upon hybridization rate was explored with a generalized linear model for both *H. annuus* and *H. petiolaris*. Both main effects of soil moisture and shelter were non-significant (Table 1). Note, however, that the marginally significant interaction between average soil moisture and shelter ($P = 0.0967$, $R^2 = 0.208$) suggests a trend for a decrease in hybridization rate with increasing moisture when no shelter is present, but no trend when a shelter is present (Fig. 7). For *H. petiolaris* maternal plants, average soil moisture ($\chi^2 = 0.5009$, $P = 0.4791$) shelter ($P = 0.0768$), and their interaction ($\chi^2 = 0.2739$, $P = 0.6007$) did not significantly influence hybridization rates. In order to investigate the hypothesis that early season moisture (as opposed to season-wide average soil moisture, investigated above) might better predict hybridization rate, we repeated the analyses using average soil moisture measurements from the first five (July 27 – Aug. 24), four (July 27 – Aug. 7), and three (July 27 – Aug. 3) moisture sampling dates as predictor variables, respectively. The results did not differ qualitatively from the model using the season-wide average soil moisture (data not shown).

Can hybridization rate be predicted by the relative flowering intensity of the two parental species? --- Hybridization rates of *H. annuus* and *H. petiolaris* can be predicted by observing their relative flowering intensity (RFI); these relationships are consistent across rainfall treatments for both species (RFI*Treatment: *H. annuus* $F_{3,94} = 0.72$, $P = 0.5416$; Treatment: *H. petiolaris* $\chi^2 = 2.8888$, $P = 0.4091$). As RFI increases (more *H. annuus* plants are flowering relative to *H. petiolaris* plants) the hybridization rate of *H. petiolaris* increases ($\chi^2 = 3.8505$ $P = 0.0497$; Fig 8B). Likewise, as RFI decreases the hybridization rate of *H. annuus* significantly increases due to the same phenological mechanism ($F_{1,98} = 5.00$, $P = 0.0277$, $R^2 = 0.033$; Fig. 8A). Thus, for each species, hybridization rates increase when its flowers are relatively scarce.

Consistent with the finding that our rainfall manipulation treatments did not influence hybridization rates (above), RFI did not differ among treatments (RFI = treatment: $F_{3,130} = 0.11$, $P = 0.9515$) and was not influenced by soil moisture (RFI = soil moisture: $F_{1,34} = 0.21$, $P = 0.8375$) .

DISCUSSION

While hybridization rates did not differ significantly between our dry, intermediate, and wet rainfall manipulation treatments for either parental species, we did find evidence within one treatment of a trend for decreasing *H. annuus* hybridization rate with increasing soil moisture. This treatment (control open) was the sole treatment without a rainout shelter. Furthermore, we found evidence that shelters may increase the hybridization rate even when soil moisture does not differ (control shelter vs. control open). These two lines of evidence suggest that shelters influenced hybridization rates

independently of their direct effects on rainfall. Thus, non-target effects of shelters may have altered the dynamics of plants and/or their pollinators to obscure 'normal' effects of precipitation on hybridization rates (discussed below). We also found that relative flowering intensity (RFI) (the ratio of open *H. annuus* inflorescences to open *H. petiolaris* inflorescences) predicted hybridization rates between the two species, indicating that gene flow was directed from the species with more open inflorescences to the species with fewer open inflorescences. Typically, pollen movement from the more abundant species to the less abundant species has been seen in *Helianthus* as well as in other angiosperm systems (Kane 2009 Carney et al. 1994, Rieseberg 1995), but has not been previously demonstrated in an experimental context where hybridization rates were quantified in response to environmental manipulation. This result is exciting because, despite a lack of response in hybridization rates between *H. annuus* and *H. petiolaris* to the rainfall treatments, it is now clear *how* the environment can influence the hybridization rates of flowering plants.

Effect of rain-out shelters on hybridization --- We detected a trend of increased rates of hybridization in control shelter plots compared to control open plots in *H. annuus* maternal plants (Tukey's HSD test, $t_{1,24} = -2.50$, $P = 0.070$), however average soil moisture did not differ between them (Tukey's HSD test, $t_{1,32} = 1.72$, $P = 0.3324$). Additionally, we also detected an influence of shelter on hybridization rates in *H. petiolaris* maternal plants ($P = 0.0768$). Although we are unaware of the exact mechanisms that may have contributed to the influence of shelters on hybridization rates independent of the intended effects of the rainfall treatments, here we offer a few possible explanations. Shelters could have affected hybridization rates by altering the

microclimate experienced by both flowers and pollinators. First, the polycarbonate roofing may have reduced overall airflow and increased temperatures directly beneath the shelter; higher temperatures are known to alter pollinator visitation and preference (Whitney et al. 2008, Norgate et al. 2010). Second, floral humidity has been positively correlated with nectar reward (Arx et al. 2012). Therefore, it seems possible that pollinators may have also preferred the relatively higher amounts of humidity that could be trapped beneath shelters due to evapotranspiration from the plants and the evaporation of moisture from the soil. This preference for higher soil moisture may have biased pollinators to spend more time foraging in sheltered plots, thereby increasing the probability of interspecific pollen exchange between the species. The use of shelters to manipulate rainfall are common in studies examining the effects of soil moisture on natural systems (Svejcar et al. 1999, Lensing and Wise 2007, Lucas et al. 2008, Miranda et al. 2009). However, given the likely strong non-target effects of shelters on hybridization rates in our study, we suggest caution in the use of rain-out shelters in future studies examining the role of precipitation on plant mating systems.

Mechanism underlying the trend for decreasing hybridization rates with increasing soil moisture --- In this study, we detected a trend for a reduction in *H. annuus* hybridization rates as soil moisture increased in plots without a shelter (n = 9). The mechanism is unlikely to reflect an effect of soil moisture upon phenology, per se because RFI was not influenced by average soil moisture ($t_{1,34} = 0.21$ $P = 0.8375$). Instead, we hypothesize that the mechanism involves a relationship between soil moisture and pollinator behavior. Specifically, flower visitors tended to exhibit more inter-specific floral movements in plants grown under dry conditions than plants grown under wet

conditions (*unpublished data*).

Possible reasons why rainfall treatments did not alter hybridization rates --- We did not observe a change in gene flow patterns between *H. annuus* and *H. petiolaris* due to our soil moisture manipulation treatments. One possible explanation for this finding is that the phenologies of *H. annuus* and *H. petiolaris* are not sensitive to large changes in soil moisture; however, we do not believe this to be the case based upon what we know about the ecology of these species (Heiser 1961). A more likely explanation, in addition to the possible non-target effects of the shelters, was the use of seedlings in this experiment as opposed to planting seeds directly. Seedlings may not be particularly sensitive to changes in soil moisture, however small variations in soil content can strongly influence seed germination rates, as it is known to determine germination rates and success in other closely related species (Van Auken 2001). Species specific germination rates could more drastically alter flowering patterns throughout the season, and given the relationship between relative flowering intensity and hybridization rate, may result in either increases or decreases in genetic exchange.

Potential consequences of changes in patterns of gene flow between species in response to climate change --- In light of the trend that hybridization decreased with increasing soil moisture in one of our experimental treatments, climate change may influence patterns of gene flow between *H. annuus* and *H. petiolaris* in the future. As the climate warms, periods of prolonged drought along with increases in spring and winter rainfall are predicted to occur, especially in the American Midwest where these species are abundant (Mishra et al. 2010). We predict that sequential drought years could cause

an increase in interspecific gene flow from *H. petiolaris* to *H. annuus* maternal plants. In fact, recent studies examining contemporary gene flow between these species have revealed similar patterns (i.e., pollen movement from *H. petiolaris* to *H. annuus*) in contrast to more ancient patterns that were largely either bidirectional or with a slight *H. annuus* to *H. petiolaris* bias (Strasburg and Rieseberg 2008).

Alterations in gene flow between *H. annuus* and *H. petiolaris* could have multiple possible outcomes. Consequences of past hybridization events within the genus *Helianthus* have been both constructive and destructive (Rieseberg 2006). Three separate instances of homoploid hybrid speciation have been observed within this group (Abbott 1992, Donovan et al. 2010). In contrast, there is one putative example of reverse speciation (genetic extinction of an evolutionary lineage due to high rates of unidirectional hybridization) (Seehausen et al. 2008) between *H. annuus* and *H. bolanderi* (Carney et al. 2000), whose ranges and ecological requirements largely overlapped. However, species boundaries such as those between *H. annuus* and *H. petiolaris* may be robust to changes in gene flow patterns due to large effective population sizes (Strasburg et al. 2011) and the maintenance of species integrity in the face of gene flow (Rieseberg et al. 1995, Strasburg and Rieseberg 2008).

For species that have restricted ranges and low genetic diversity (Ellstrand et al. 1989), hybridization could greatly impact their evolutionary trajectories and long-term persistence (Stebbins 1959, Arenas et al. 2012). For instance, genetic swamping, when a more abundant species overwhelms a less abundant species via large amounts of interspecific gene flow, can effectively eliminate genetic lineages because hybrid

offspring are often less fit than their parents (Seehausen et al. 2008, Beatty et al. 2010). Extinctions via a loss of genetic integrity have occurred across taxa as a consequence of increased rates of genetic exchange (Thomas et al. 2004, Buggs and Pannell 2006, Beatty et al. 2010), which may become more common as species interactions are further augmented by a changing environment (Crispo et al. 2011). As seen in the *Helianthus* complex, speciation and adaptive radiation may also be a potential outcome of interspecific gene flow, which can happen if hybrids are fit and able to occupy a different ecological niche than the parental taxa (Seehausen 2004, Rieseberg 2006). The speciation process may even occur in a single generation, as seen in instances of polyploidy, where offspring are immediately reproductively isolated from both parents (Eckenwalder and Brown 1986, Wood et al. 2009). Lastly, hybridization may also rescue populations from decline by infusing otherwise genetically depauperate populations with potentially adaptive genetic diversity (Hughes et al. 2008, Valdiani et al. 2012). Experimental manipulations of gene flow between populations adapted to similar thermal environments have shown that increased genetic diversity can improve fitness of outcrossed individuals compared to inbred individuals (Sexton et al. 2011). Therefore, adaptation facilitated by recombination may be an important way in which natural populations persist despite warmer average temperatures (Franks and Weis 2009, Hoffmann and Sgrò 2011).

Future directions --- Given the myriad demographic and evolutionary ramifications of altered patterns of gene flow, it is critical to quantify genetic exchange between species as they respond to a quickly changing environment. Future studies pairing observations of climate change effects on hybrid formation and on hybrid persistence (Campbell & Wendlandt *in review*) in the same system are needed to

accurately predict how climate change will impact rates of interspecific gene flow. Further, the use of relative flowering intensity (RFI) in this study was an informative metric that predicted rates of hybridization between *H. annuus* and *H. petiolaris.* RFI, along with other estimators of hybridization such as interspecific pollinator movements (Campbell et al. 2002), could be used in the field as cost-effective ways to estimate rates of hybridization between flowering plants as their phenologies respond to climate change.

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Fig. 1. Diagram of half of the plot layout. P symbolizes *H. petiolaris* individuals and A symbolizes *H. annuus* individuals. Numerical subscripts indicate individual plants.

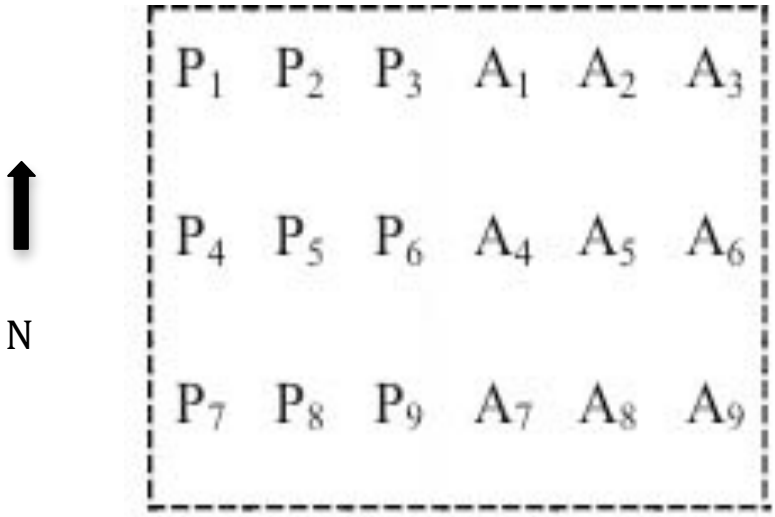

Fig. 2. Rain-out shelters used to capture rainfall. Rainfall hit the transparent roofing, was caught in the rain gutter, and drained into a rain barrel. The rain was then redistributed using a 4-hp water pump and tractor.

Fig. 3. The fraction of F_1 *H. annuus* individuals confirmed to be of hybrid status as a function of leaf abaxial trichome density. No individuals possessing >50 abaxial trichomes/mm^2 were hybrid.

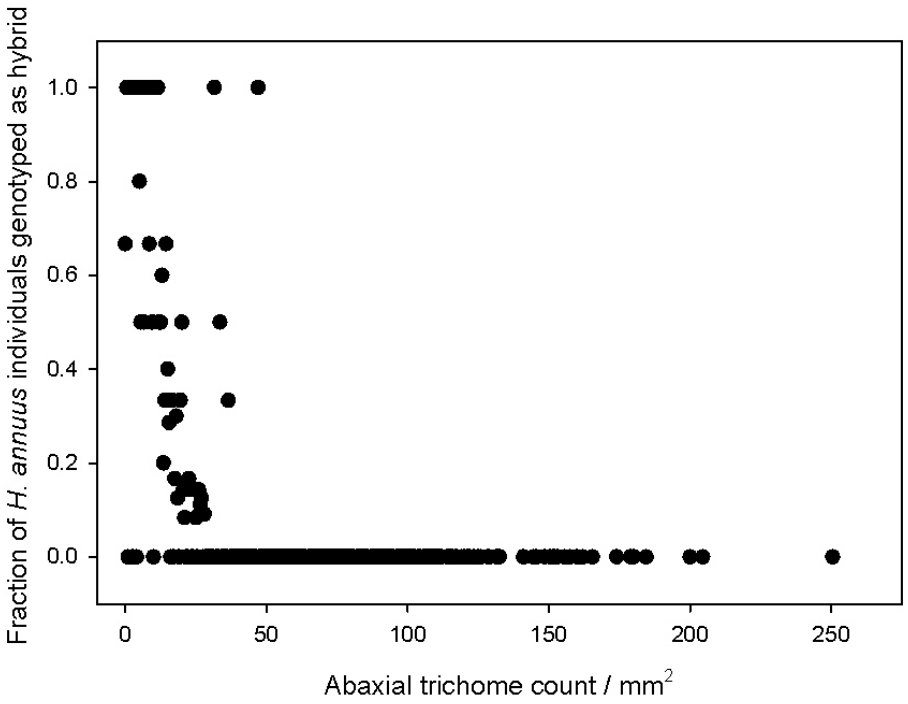

Fig. 4. The external transcribed space region of the 18s rDNA (ETS) visualized using gel electrophoresis. Hybrids (H) possess two bands while each parent expresses a single unique band (P, *H. petiolaris*; A, *H. annuus*).

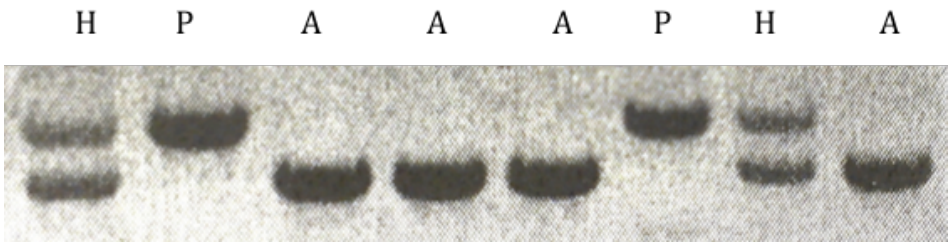

Fig. 5. Average soil moisture across the season for each rainfall manipulation treatment.

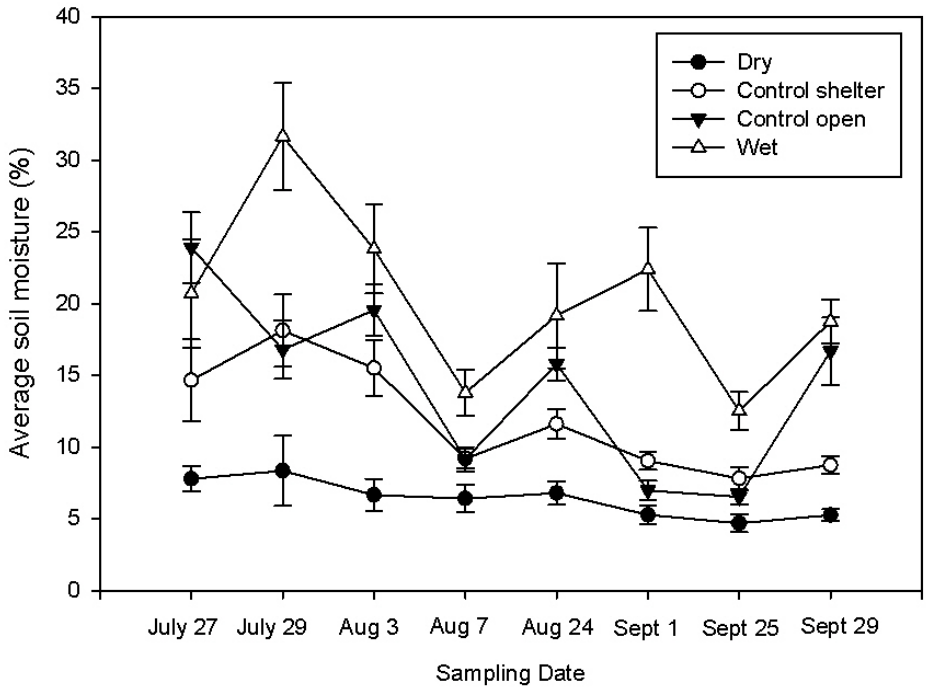

Fig 6. Average hybridization rate (fraction of progeny that were hybrids) for each species across four rainfall treatments.

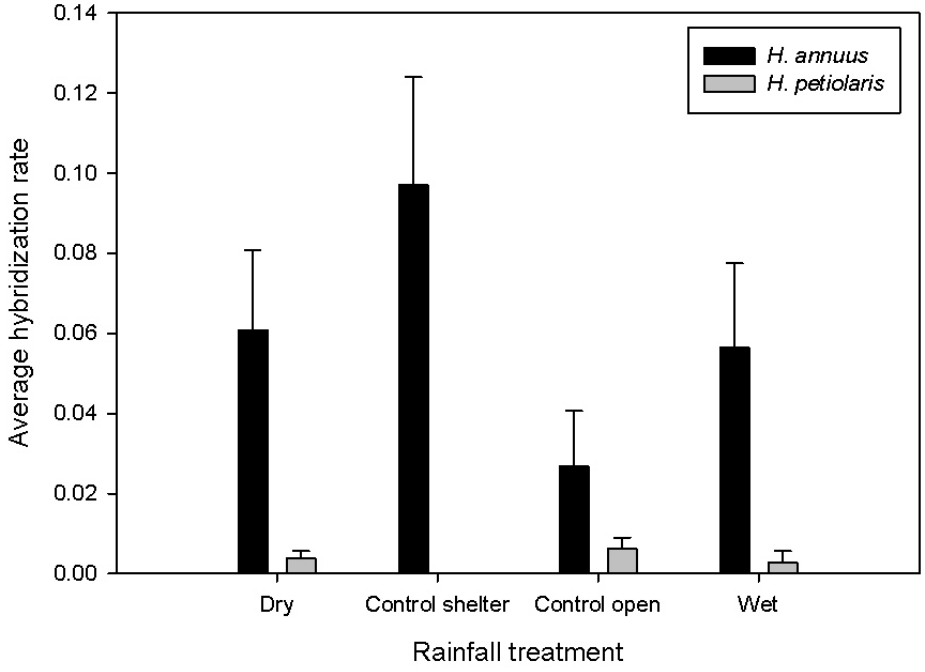

Fig. 7. The relationship between soil moisture and average hybridization rate of *H. annuus* plants in plots with (n = 27) and without (n = 9) rainout shelters.

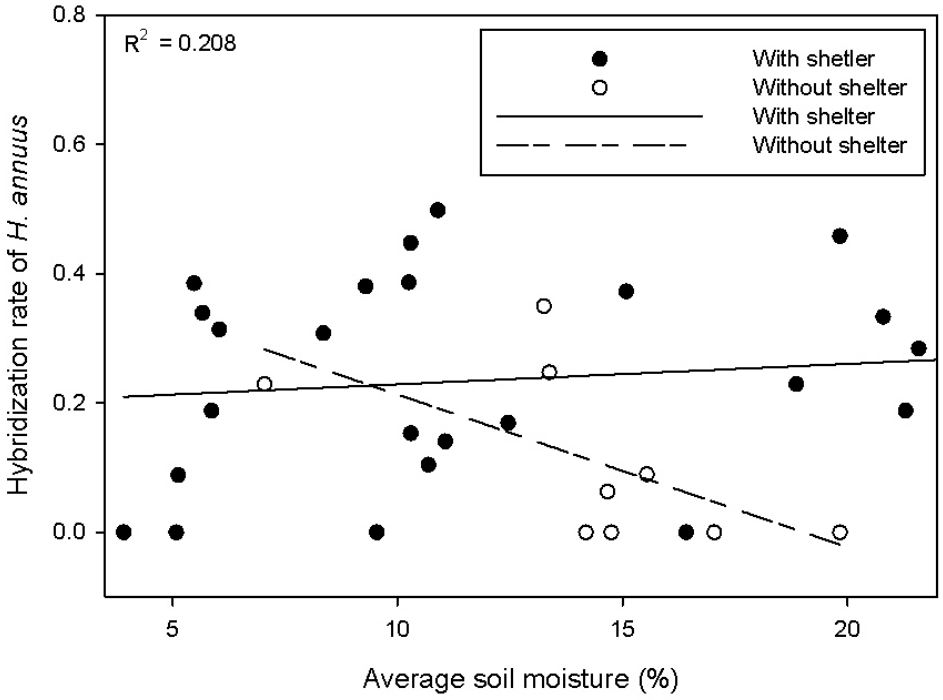

Fig 8. Hybridization rates of *H. annuus* (A) and *H. petiolaris* (B) predicted by relative flowering intensity (RFI). Each symbol represents a single plot on one of four sampling dates. Plot 16 was removed from the analysis due to complete *H. peitolaris* mortality in this plot. In B, one treatment out of four was plotted to demonstrate the slope of the curve, but for simplicity the other three treatments are not shown.

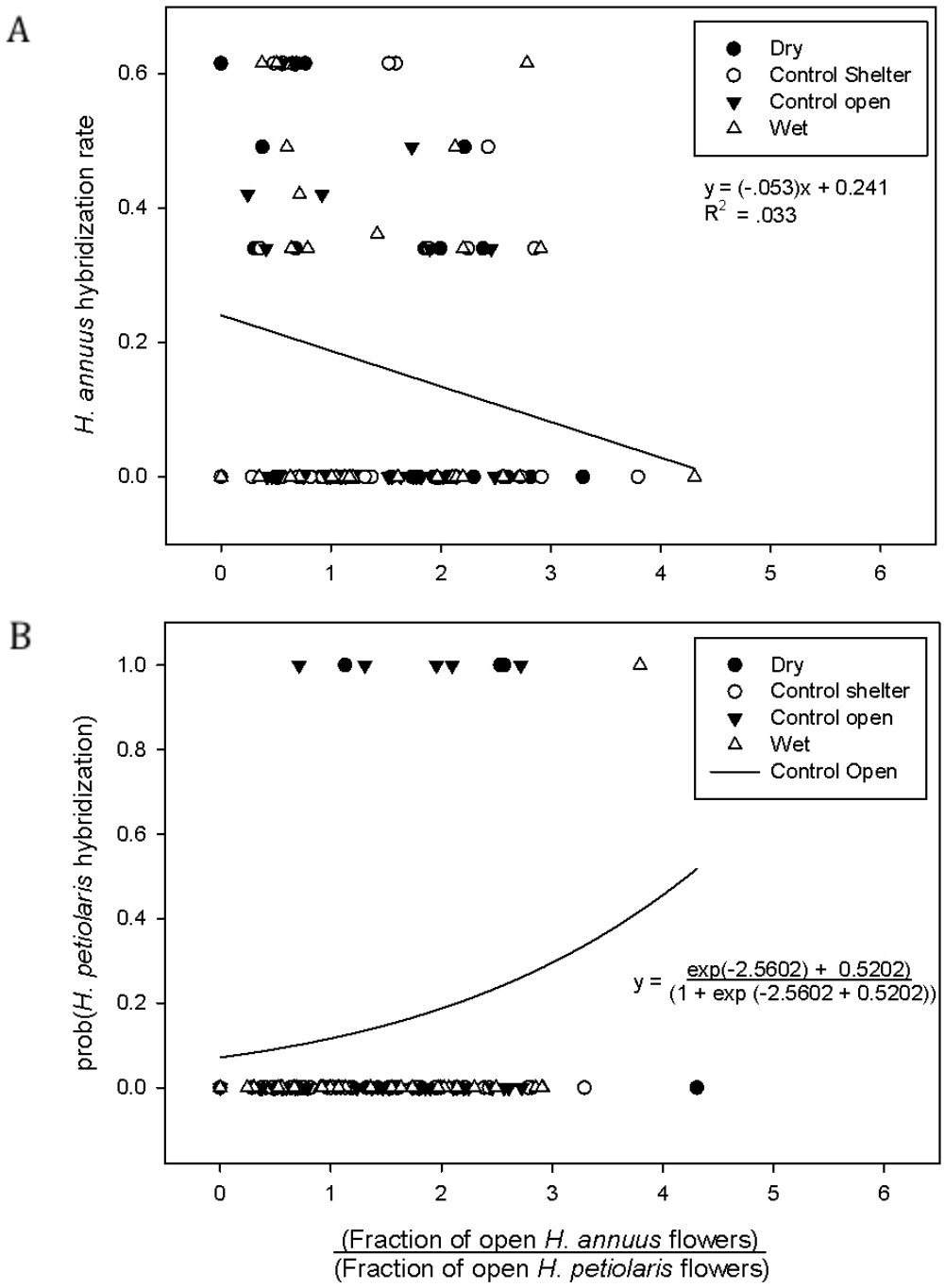

Table 1. Linear model analyzing the effect of average soil moisture and shelter presence/absence on *H. annuus* hybridization rate.

Effect	df	MS	f	P(f)
Average soil moisture	1, 31	0.0359	1.71	0.2006
Shelter	1, 31	0.0253	1.2	0.2812
Average soil moist * Shelter	1, 31	0.0617	2.93	0.0967