FINAL PERFORMANCE REPORT

As Required by

THE ENDANGERED SPECIES PROGRAM

TEXAS

Grant No. TX E-110-R-2

F11AP00449

Endangered and Threatened Species Conservation

Reproductive Biology, Genetics and Ecology of South Texas Ambrosia: Implications for the Management, Recovery and Reintroduction

Prepared by:

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WILDLIFE

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25 January 2016

FINAL REPORT

STATE: ____Texas_______________ GRANT NUMBER: ___TX E-110-R-2__

GRANT TITLE: Reproductive Biology, Genetics and Ecology of South Texas Ambrosia: Implications for the Management, Recovery and Reintroduction

REPORTING PERIOD: ____1 August 2011 to 31 July 2015

OBJECTIVE(S). To acquire the basic genetic, ecological, and reproductive data currently lacking on *Ambrosia cheiranthifolia* necessary to manage of extant populations scientifically and to write an evidence-based protocol for future reintroduction efforts.

Segment Objectives:

Tasks:
July 1, 2011-August 30, 2011
-Visit accessible sites
-Collect samples for preliminary genetic study; extract DNA and start optimizing microsatellites

September 1, 2011-August 30, 2012
-Complete preliminary genetic studies
-Begin population monitoring, including physiological ecology studies
-Begin soils study
-Choose sites for management study
-Begin data analyses and manuscript preparation as appropriate

September 1, 2012-August 30. 2013
-Begin full genetic diversity study
-Continue population monitoring
-Complete soils study
-Continue data analyses and manuscript preparation as appropriate
-Begin management study

September 1, 2013-June 30, 2014
-Complete full genetic diversity study
-Complete population monitoring
-Complete management study
-Complete data analyses and manuscript preparation
-Complete Reintroduction Protocol

Significant Deviations:

Project was extended to 31 July 2015 to accommodate more time needed to complete research and to allow for change in Principal Investigator (now Dr. Dave Grise). Final report was delayed due to problems obtaining results for genomic sequencing analyses. The current report still lacks these data however the PIs pledge to provide those data as soon as they become available. Please see pp 80-81 of the attached report for further explanation.

Summary Of Progress:

Please see Attachment A.
Location: Nueces and Kleberg Counties, Texas.

Cost: ___Costs were not available at time of this report, they will be available upon completion of the Final Report and conclusion of the project.__

Prepared by: _Craig Farquhar______________ Date: _25 January 2016

Approved by: ________________________________ Date:_____25 January2016
C. Craig Farquhar

ATTACHMENT A

Section 6 Final Report

Reproductive Biology, Genetics and Ecology of South Texas Ambrosia: Implications for the Management, Recovery and Reintroduction

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Abstract

South Texas Ambrosia (*Ambrosia cheiranthifolia*) is monoecious, federally and state endangered plant that is endemic to the coastal prairie region of South Texas. Crucial information on its genetics, reproductive biology, and habitat preferences needed to produce a successful reintroduction protocol have been lacking. The goal of this study was to acquire the basic genetic, ecological, and reproductive data to manage extant populations scientifically and to write an evidence-based protocol for future reintroduction efforts.

Survey and mapping results indicate that stem densities vary considerably across sites. Although mapped polygon sizes were somewhat stable, densities within some polygons have decreased. Flowering, as well as its phenology, also varied across sites and in some sites was impacted by mowing: mowing during flowering removed all inflorescences in one polygon in 2013. One troubling result was that we did not observe fruits during the study period at any site. Sixteen soil characteristics did not differ statistically between areas with Ambrosia and nearby areas without Ambrosia ($P = 0.51$) but did differ statistically among populations locations ($P < 0.001$). However, no clear patterns emerge that might explain differences in stem density among sites.

Genetic studies with microsatellite markers indicate that Ambrosia patches may be single clones but are composed of multiple interdigitating clones in some locations. We found 31 clones, some of which are widely distributed; however, clones appear to be highly related. These results indicate that some sexual reproduction occurs in this species or at least has occurred. The expected genomic sequencing data should clarify these issues.

Management impact studies of the effects of prescribed burning and mowing were also conducted. We found that burning effectively reduced grass cover and Ambrosia rapidly resprouted, grew, and flowered after the fire. However we did not find strong differences in changes in stem density between plots that were burned and those that were not; however, flowering may have increased in burned plots. Mowing also did not produce strong differences in stem density or flowering among mowing regimes; however, mowing that reduced Ambrosia stems may have contributed to a population crash in one site. For both studies, great heterogeneity among plots may have contributed to the lack of a strong response by Ambrosia. Based on our results, either form of management could be used to manage competitors, such as invasive grasses; however, it appears that burning may be more effective in reducing grass cover.

Introduction

South Texas Ambrosia (*Ambrosia cheiranthifolia*), hereafter simply “Ambrosia,” is a herbaceous, monoecious plant species endemic to the South Texas Coastal Prairie with an apical male inflorescence with flowers that open before the axilary female flowers (Lehman et al. 2005, Poole et al. 2007). Due to it being restricted to only a few known locations, the species was federally listed as endangered in 1994 (59 FR 43648 43652) followed by state listing in 1997 (Poole et al. 2007). As with virtually all South Texas rare species, the most basic information on the genetics, reproductive biology, soil, and habitat preferences necessary to write a scientifically based plan for successful reintroductions is lacking. Understanding reproductive and demographic processes, community ecology and dynamics, soil chemistry, habitat management effects, and the interaction of all these factors with population genetics and meta-population structure are critical components for the establishment of *ex situ* garden populations and seed banks, augmentations, reintroductions, and the eventual recovery, of Ambrosia. As Ambrosia commonly co-occurs with other listed and tracked species (e.g., *Hoffmanseggia tenella*, *Echeandia chandleri,* etc.) in short grass prairie remnants in Nueces and Kleberg County, our study has relevance to conservation efforts of these co-occurring species and the short grass prairie in which these communities occur.

Historically, the single biggest threat to rare plant species in South Texas has been habitat loss and land management changes due to agriculture and development, but the competitive and other negative ecological effects of invasive range grasses are emerging as the greatest treats to currently identified and protected populations. Simple protection of extant populations without management for invasive grass is likely to result in the eventual loss of these populations. Mowing and fire are major ways with which invasive grasses are being dealt with in Texas (e.g. Simmons et al. 2007, Tidwell et al. 2012, Havill et al. 2015), but the effects of these on Ambrosia mortality and reproduction are not well understood. Some mowing studies on NAS-Kingsville (Bush et al. 1994, Garvon 2005) suggest that mowing frequency has some effect on stem counts.

South Texas Ambrosia has a rhizomatous growth form; therefore, a given population of plants may be largely of clonal origin. How much recruitment is asexual (vegetative) or sexual is unknown. Studies of other rare plants with clonal growth capabilities, such as Mead's Milkweed, have shown that management practices have an impact on the genetic structure of the population by presumably favoring asexual or sexual reproduction (Tecic et al. 1998). Is sexual or asexual reproduction more important in Ambrosia populations? For example, are new stems products of sexual or asexual reproduction? Are certain genotypes more common in "new" areas, or conversely are certain genotypes being lost at greater rates than others over time as patches shrink or retreat from an area? The answers to these questions have important implications to conservation and management efforts.

To make informed decisions regarding management choices, land acquisition or conservation easements, *ex situ* breeding programs and re-introductions, some understanding of the population dynamics and genetic structure of the species and its sub-populations is necessary (Frankham et al. 2002, Olfelt et al. 2001, Wolf and Sinclair 1997). New powerful methods based on next-generation sequencing techniques have recently been introduced that are both cost effective and applicable to natural populations of non-model organisms such as Ambrosia (Toonen et al. 2013, Puritz et al. 2014a,b). Here we employ these methods as well as results from more traditional microsatellite markers and concomitant ecological studies to make recommendations concerning priorities for the *in situ* and *ex situ* conservation efforts for South Texas Ambrosia and to elucidate the reproductive biology and metapopulation structure, and to suggest optimal strategies for reintroduction and recovery efforts.

Objective

To acquire the basic genetic, ecological, and reproductive data currently lacking on *Ambrosia cheiranthifolia* necessary to manage of extant populations scientifically and to write an evidence-based protocol for future reintroduction efforts.

Locations

We visited eight locations encompassing 25 occurrences, including two thought to be extirpated, of *Ambrosia cheiranthifolia* (hereafter Ambrosia) during the course of this study. These areas and activities at each location from 2012-2015 are listed in Table 1, and locations are indicated on the map in Figure 1.

Methods

Ecology and Demography

In 2012, we visited all sites listed as extant by Hempel and Overath (2009) and several that were listed as extirpated or unknown (Table 1). We began annual (spring/early summer) and fall flowering surveys in 2013.

Annual Surveys and Fall Flowering Surveys

Polygon delineation and survey methodology: In 2011 and 2012, members of the Overath lab participated in the USFWS annual survey of Ambrosia at the Naval Air Station—Kingsville (NAS-K) to learn their methods. We adapted these methods for our own surveys. In May-June 2013 we visited the accessible extant Ambrosia patches at Robstown Park, St. James Cemetery, and the two sites on US 77 near the Kleberg-Nueces County line (Table 1, Figure 2) to conduct an "annual survey". At each location we used flags to delineate the edge of Ambrosia patches; carefully searching for stems, we followed the USFWS criterion that any stem within 2 m of another stem was in the same patch or polygon. Once a polygon was delineated with flags, the polygon perimeter was walked with a 2008 Trimble GeoExplorer XT, loaned to us by the Geospatial Sciences Lab at the Harte Research Institute for Gulf of Mexico Studies, to record its outline. Upon return to the lab, these data were downloaded from the Trimble and loaded into ARC-GIS Ver. 10, and a map was generated for each location.

Once a polygon was delineated and traced, a transect composed of rope marked at 1-m intervals was laid down long its main axis. Random numbers were drawn to choose coordinates along the transect to place a 1-m^2 quadrat and all stems within the quadrat were counted. For transects > 10 m long, 10 quadrats were sampled; whereas <10 transects were sampled for shorter transects (Table 2). In the few polygons less than 1-m^2 in size, all stems in the polygon were counted. GPS coordinates of transects ends were recorded with a GARMIN *GPSmap* 60CSx to assist in relocation for subsequent surveys.

In November 2013, all three locations were visited again to perform a fall flowering survey, and 1-m^2 quadrats were randomly chosen as described for the June survey. Each stem in the quadrat was counted and its reproductive status (male inflorescences, female inflorescences, both, or none) recorded. However, at St. James Cemetery the 0.25-m^2 semi-permanent plots were surveyed instead, as described below in the "Management Impacts" section. Polygons at Robstown Park and St. James Cemetery were delineated and surveyed again in June 2014. Due to construction on US 77, those sites were not accessible. A fall flowering survey could not be conducted at these locations in 2014 because flowering had ended before we could perform the surveys. To obtain additional data, a 2015 fall flowering survey was conducted at Robstown Park (Co. Rd 73 polygons only) and St. James Cemetery (polygons 1 and 2 only). Also in 2015, Ambrosia was found again at Bishop City Park (albeit in a different location than that it was known from previously); therefore, we performed a fall flowering survey in two of the patches representing the types of areas Ambrosia occurs in this location: a cut-grass path or “road” area and a grassy area that appears to be unmowed (or at least not mowed frequently).

Physiological Ecology

Physiological measures such as photosynthetic rate and stomatal conductance will also be estimated twice a year during demographic monitoring. We have a LI-6400 photosynthesis system that can be used for this purpose. These measurements can be done non-destructively; however, we will need to remove the measured leaf from plants with very small leaves. Therefore, plants with larger leaves will be preferentially measured. Photosynthetic rate will be used as an indication of plant health and stomatal conductance is a measure of water status. These measurements can be compared across sites and over time in relation to environmental conditions, such as rainfall.

GPS/GIS

2008 Trimble GeoExplorer XT, loaned to us by the Geospatial Sciences Lab at the Harte Research Institute for Gulf of Mexico Studies was used to obtain fine scale outline maps of patches during annual surveys as described above, so that meter level changes in patch extent and location might be documented for future reference. These maps will also be used in a GIS analysis of habitat characteristics and soils.

Soils

Sample collection: Soil samples were gathered within Ambrosia population areas and ca. 10 m from the edge of the population/polygon at 11 sites (Table 3) and sent via 2-day air to the Cornell Nutrient Analysis Lab at Cornell University. Following directions provided by the lab, we collected approximately 1-cup samples and placed then in numbered plastic zip-lock-type bags. Modified Morgan extractable elements (see list in Table 3) were determined by ICP analysis. Percent organic matter was determined by the loss-on-ignition method. Moisture content and pH were also measured. All methods followed the National Soil Survey Center’s Soil Survey Laboratory Manual (Soil Survey Staff, 2014).

Data analysis: To determine if soil element concentrations and other soil characteristics in areas with Ambrosia differed from those without Ambrosia or between sites, we performed a MANOVA using R version 3.2.1 for Windows (The R Foundation for Statistical Computing). The model included “treatment” (Ambrosia or no Ambrosia) and site (collection location) as well as their interaction. We also generated univariate ANOVA tables for each soil characteristic from the MANOVA results. Because the Demonstration Site is not a “natural” population, we repeated this analysis without that location; however, since the results were essentially identical, we report only results for the full data set. For soil characteristics that varied significantly, we ran separate ANOVAs using the same model followed by Tukey’s multiple range test with significance of P-values corrected for multiple comparisons.

Genetics

Microsatellite Markers

Most of the microsatellite work was carried out as part of a study on Ambrosia at NAS-K funded by the Navy (Overath et al. 2013). We recount the genotyping aspect of that work here as it was also done to meet objectives of this study.

Sample collection: 80 small leaf samples were collected from 3-6 stems per large USFWS polygon) within 12 Ambrosia sites in Fall 2011 during the annual USFWS density survey. These samples were collected haphazardly but spaced out enough to ensure two samples did not come from the same small patch, which we assumed were clones (e.g., the cardinal points of a polygon where used when possible). Location was recorded as the center of the polygon rather than having a separate point for each stem, due to time and equipment constraints. In July 2012 we collected an additional 35 samples from a small patch of Ambrosia at the St. James Cemetery. Additional samples were collected from other Ambrosia sites in 2013 and 2014 but were not completely analyzed due to a switch to genomic sequencing techniques.

DNA extraction and fragment analysis: We extracted genomic DNA with the DNeasy Mini Plant Kit (Qiagen, Valencia, CA) using the standard manufacturer's protocol altered to first lyse leaf tissue as described in Matakis et al. (2011). We then genpotyped using PCR of primer pairs previous developed for the related species *Ambrosia artemisiifolia (Genton et al*. 2005; Abercrombie et al. 2009). Work conducted in the Overath lab in 2010 indicated that five of these primer pairs (*Amb*12*, Amb*82*, Ambart*04, *Ambart*09, and *Ambart*21) amplified reliably in our Ambrosia species (Overath et al. 2013). We genotyped samples using a variation of M13 labeling methods of Schuelke (2000) to add fluorescent dye to the resulting PCR fragments for detecting size in an automated sequencer. A 10µL PCR reaction was performed that included 2.75µL of sterilized molecular grade H_2O, 5.00µL of 1X Phusion® High-Fidelity PCR Master, 0.25µL of 10µM M13-tagged forward primer, 0.50µL of 10µM reverse primer, 0.50µL of fluorescent M13 (6-FAM, HEX, or NED), and 1µL of approximately 10ng of genomic DNA. Thermocycling conditions began with an initial denaturation step at 98˚C for 2 minutes, followed by 33 cycles of denaturation (98˚C for 10 seconds), annealing (30 seconds at a primer-pair-specific temperature: 64˚C for *Amb*12 and *Amb*82*,* 52˚C for *Ambart*04, 67˚C for *Ambart*09, and 64˚C for *Ambart*21), and extension (72˚C for 15 seconds). An additional 8 cycles were added to optimize the fluorescent M13 labeling (denaturation at 98˚C for 15 seconds, annealing at 53˚C for seconds, and extension at 72˚C for 30 seconds). A final extension step was included at 72˚C for 5 minutes followed be a hold at 4˚C. PCR products were analyzed by fragment analysis at Molecular Cloning Laboratories (MC Lab; www.mclab.com).

Following recommendations from Selkoe and Toonen (2006), we reran a previous sample and a control containing no DNA with each set of PCR reactions and subsequent fragment analysis. Rerunning previously genotyped individuals allowed the possibility to correct fragment sizes for inconsistencies between runs; however, in all except one run, all fragment sizes were within a few hundredths of a bp and no correction was needed. "No DNA" controls served to identify "artifact" fragments that were then ignored in the sample genotyping. All individuals were run at least twice to verify genotype. Final binning of fragment sizes and recording of genotypes was performed by one person (Overath) to reduce binning error (Selkoe and Toonen 2006).

Only those samples that had a repeatable genotype were included in the data analysis procedures described below.

Data analysis: Because the two primers that had the most variation showed evidence of polysomic inheritance (i.e., 1-4 fragments in an individual), we rescored size in bp data for each possible fragment from the variable loci as present (1) or absent (0). In other words, in order to use the most variable loci, we had to treat our data as having dominant inheritance and as if haploid rather than the codominant inheritance that characterizes microsatellites from diploid species. Because of this, traditional measures of variation such as heterozygosity and departure from Hardy-Weinberg equilibrium could not be calculated. Instead, we focused on estimating and describing clonal diversity in Ambrosia, which was an important objective for this study as it could indicate levels of asexual vs. sexual reproduction. We first trimmed the data set to exclude samples with missing data and then identified clones and clone genotypes and overall frequencies using the "Clonal" function in *GenAlEx 6.5* (Peakall and Smouse 2006, Peakall and Smouse 2012). Clone information was imported into *GenoDive* (Meirmans and Van Tienderen 2004), which is a program designed to calculate diversity measures for clonal species. Number of genotypes, effective number of genotypes (which takes sample size and eveness into account), and Nei's genotypic diversity were calculated for each of the areas. Nei's genotypic diversity, which is essentially the probability of two randomly chosen samples being different genotypes or clones, was also calculated for the data set as a whole and partitioned into within and among location components. This diversity measure is equivalent to expected heterozygosity (He) in a diploid species. We also performed 10,000 permutations of the data to test for possible differences in genotype (really clone) frequency differences among pairs of areas in *GenoDive*. This is the most appropriate way to evaluate possible genotypic differences among sample locations (Meirmans and Van Tienderen 2004). Due to the high number of comparisons (105), we used the Bonferoni correction for evaluating significance of the permutation results (Rice 1989).

To explore clone relationships we also used the "Distance" function in *GenAlEx* to calculate Euclidean distances between clones. *GenAlEx* calculates this measure using the method of Huff et al. (1993), which totals the number of fragment differences between pairs of clones. We then constructed a phenogram from the resulting distance matrix using the UPGMA clustering algorithm in *NTSYS 2.2* (Rohlf 1997). We also included samples collected in 2010 along transects in two other areas of NAS-K (USFWS areas 11 and 14) in these analyses and to examine the arrangement of clones by graphing clone locations (latitude and longitude) in *GenAlEx*.

Additional primer optimization: We also did extensive testing with two other types of polymerase with different activity than the Phusion polymerase we used to genotype Ambrosia as described above. GoTaq® Green Master Mix, which is a less specific polymerase that we hoped would prove useful in cross-species primer amplification, and Pfu polymerase, a high-fidelity polymerase similar to Phusion, but with different activity. Using manufacturer's recommended PCR profiles as a starting point, we performed the same optimization procedures as described in Overath et al. (2013) for primer pairs *Amb*16 and *Amb*30 (Genton et al. 2005) and *Ambart*18 and *Ambart*27 (Chun et al. 2009), which did not amplify or amplified unreliably with Phusion. Unfortunately, we were never able to reliably amplify any of these loci, prompting us to turn to other, newer methods based on next-generation sequencing techniques.

Genomic sequencing

ezRad protocol: New approaches are now allowing for affordable genomic sequencing of non-model species, including those with large genomes. One such approach, restriction site associated DNA sequencing or RADseq, which does not sequence the whole genome, but instead focuses on pieces of the genome near restriction sites (Toonen et al. 2013, Puritz et al. 2014a). One such method, ezRAD, has been developed to allow the discovery of large numbers of single nucleotide polymorphisms (SNPs) in natural populations of non-model species with few genetic resources available (Toonen et al. 2013). This approach can generate hundreds of markers. One of the developers of this new approach is a colleague at Texas A&M Unviersity—Corpus Christi, Dr. Chris Bird. As part of the mowing management impacts study described below, we collected leaf tissue from every stem in a subset of the semi-permanent plots (two plots from two sites from each of the three mowing regimes) we established in July 2014 and extracted DNA as described above. DNA samples were handed over to the Genomics Core Facility at TAMU-CC and stored at -80˚C. The Genomics Core Lab used DNA electrophoresis to check the quality of the DNAs and chose approximately the best 20 from each plot for analysis. Before library preparation, the heart of this process, began DNA was further purified using SPRI beads (Tooner et al. 2013). The next step in the process was DNA digestion by restriction enzymes are used to cut the DNA as described in Toonen et al. (2013) to prepare a DNA library. Specific adapters that essentially “barcode” the fragments were ligated to the fragments using the Illumina TruSeq DNA kit. Fragments of size 400-500bp were then selected by gel electrophoresis and extracted from the gel. These fragments were then validated and sent to the University of Texas at Austin Sequencing Facility for Illumina sequencing.

Bioinfomatic analyses: Resulting sequencing reads must be run through a bioinformatics pipeline to filter reads and assemble fragments into contigs and read a map. Following this, SNPs are called and then questionable SNPs are filtered out. The TAMU-CC Genomics Core Facility uses the dDocent pipeline (Puritz et al. 2014b) for this purpose and use it will call and filter SNPs from Ambrosia sequences. We will follow this with basic population genetics analyses to estimate levels of variation and potential effects of mowing using *GenAlEx 6.5* (Peakall and Smouse 2006, Peakall and Smouse 2012). If possible, we will also use GenoDive (Meirmans and Van Tienderen 2004) to identify clones and estimate clone number and diversity. All diversity measures will be compared among sites and mowing regimes using a nested ANOVA design as described for ecological data in the Management Impacts section below.

Management Impacts

We examined the effects of prescribed burning and mowing on Ambrosia in separate studies. For both sets of studies we established 0.5 m X 0.5 m semi-permanent plots in Ambrosia polygons. To establish these plots we first set up a transect along the main axis of the chosen polygon and located a plot by randomly choosing a distance along the transect and then flipping a coin to chose which side of the transect to locate the plot. If the random location did not contain Ambrosia stems, we chose another plot. Plot corners and GPS coordinates of transect ends were recorded using a GARMIN *GPSmap* 60CSx, and plot corners were marked as discussed below. We conducted surveys using a 0.5 m X 0.5 m quadrat made from PVC pipe and marked at 1-cm intervals and meter sticks. Plant heights were measured with a measuring tape. In order to have consistent estimates, one of us (Overath) estimated percent cover of Ambrosia, grasses, forbs, and bare ground for each plot from photographs taken during the surveys noted below. Quadrat markings were used to assist with the percent cover estimation. All statistical tests were performed using R version 3.2.1 for Windows (The R Foundation for Statistical Computing).

Effects of Prescribed Burning

Plot Establishment & Surveys: The burn study was conducted at the St. James Cemetery in Bishop, TX (Figure 3) as an extension of a study of the effects of burning and other practices on slender rush pea (*Hoffmannseggia tenella*) conducted by Dr. Sandra Rideout-Hanzak of Texas A & M University—Kingsville at the same site. We established five semi-permanent plots as described above in Ambrosia polygon 1 and another five in polygon 2 (Figure 3). The four corners of each plot were marked with a numbered metal tag, and a flag was placed in each plot. The x-y coordinates of each individual stem were recorded on July 29, 2013. Dr. Rideout-Hanzak burned an area of the cemetery that included polygon 1 on August 6, 2013. We visited the site for the following three weeks to note plant reemergence after the fire in the burned area. We then resurveyed the plots in both the burned and not burned areas in September 2013, October 2013, November 2013, January 2014, March 2014, May 2014 and August 2014. Photos were taken of each plot during the July 2013, September 2013, and August 2014 surveys. As part of the November 2013 survey, the reproductive status (male inflorescences, female inflorescences, none, or both) of each stem was also recorded. Height of each stem (in cm) was recorded as part of the May and August 2014 surveys.

We had planned to record reproductive status again in 2014, but collecting data for the mowing study described below took much longer than planned due to weather and availability of personnel and reproduction at this site was done before we could conduct the survey. However, we did collect additional reproductive data for this site in November 2015 in polygons 1 and 2 using the same 1-m quadrats we used for annual surveys because we could not find many of the semi-permanent plots, especially in the unburned (polygon 2) area.

Data analysis: To test for differences between the burn treatments for Ambrosia stem density and percent cover, we first calculated the change after the burn between post- and pre-burn by subtracting the number of stems per plot (or percent cover, as appropriate) obtained during the pre-burn survey in July 2013 for each survey date. We then tested for differences in the mean change between the burned and the unburned plots using ANOVA with the factors burn treatment, time (survey date), and their interaction. Focusing on change in these values after the burn treatment is the most appropriate approach because Ambrosia stem density and percent cover were not very similar between the burned and the unburned areas before the burn. To see if the burn treatment influenced grass and forb cover, change in percent cover of these groups were calculated and tested the same way as the percent Ambrosia cover data.

Differences in mean plant height were tested using this same ANOVA model. Percent flowering was tested in a one-way ANOVA with the single factor “treatment” due to differences in the quadrat size used for the November 2013 and 2015 surveys. Both the percent flowering and the percent cover data were arc-sine transformed for statistical analyses.

Effects of Mowing

Plot Establishment & Surveys: With the assistance of NAS-K staff, we chose nine areas in July 2014 to represent the three mowing regimes in practice at the base: not mowed, tractor mowed, and zero-turn mowed (Figure 4 and Table 4). Within each site we delineated an Ambrosia polygon as for the annual surveys described above and randomly chose five plots. The ends of the transect and corners of each plot were marked with lawn fabric staples. Three of the corner staples were painted bright pink and one, the 0,0 or SW corner, was painted blue to orient the quadrat properly during surveys. For plots in sites that were not mowed, the wooden stakes used to attach the transect rope were flagged with flagging tape and left in place. Plots were surveyed in July 2014 and November-December 2014. During each survey we recorded the x-y co-ordinates, height, and number of branches of each stem. Reproductive status, as noted above, was recorded for each stem in the November-December 2014 survey. A photo was taken of each surveyed plot during the July 2014 and July 2015 surveys. Unfortunately, one of the not mowed sites (N3) had to be mowed after the initial July 2014 survey and was dropped from the study. In July 2015, we attempted to survey again but most of the transects and plots could not be re-found after extensive searching, therefore, only number of stems was recorded for this survey in the plots that could be found. To collect additional reproductive data in November 2015, using the same 1-m quadrats we used for annual surveys at all sites except the not mowed site that was dropped from the study.

Data analysis: Because we had five plots in each site and three sites in each treatment (at least for the first census), the design of this study is hierarchical or nested—plot is nested in site, which is nested in mowing regime. This type of design is most appropriate when faced with high environmental heterogeneity and a limited number of possible replicates as in a study such as this. Taking this nesting into account as part of the ANOVA model counteracts possibility of the high variability masking the effects of the treatment or factor (Logan 2010). Therefore, we used this nested ANOVA model to test for differences among mowing regimes for stem density, plant height, number of branches per stem, and percent cover of Ambrosia, grasses, and forbs. Due to the loss of one of the not mowed sites, we analyzed the July 2014 and November-December 2014 data in separate ANOVAs. In addition, due to many zeros in the reproductive data because in some cases the plots contained no stems and in others they contained no flowering stems, we could not statistically analyze differences in percent flowering among mowing regimes.

Results and Discussion

Ecology and Demography

During our 2012 site visits, we observed Ambrosia stems at all extant sites except the SB (east right of way) location on US 77 and the Robstown County Park location along Co. Rd. 73 (Table 1). Hempel noted several different impacts (Roundup overspray from adjacent field in 2008 and disturbance due adjacent fence repair in 2009) for the US 77 SB site and expressed concern over its survival (Hempel and Overath 2009). The Robstown County Park location was first reported in 2006 but not seen again until 2009 (Hempel and Overath 2009) However, we found and surveyed Ambrosia at both of these locations in 2013. Perhaps rainfall in April and May 2013 was enough to encourage these plants to resprout. Data from the closest NOAA/NCEI station that reports both temperature and precipitation, Kingsville, TX, indicates that total rainfall was much greater in April and somewhat greater in May in 2013 than in 2012 (Figure 7).

We could not find Ambrosia at the two extirpated/unknown sites (Bishop City Park and near Violet, TX) during these 2012 site visits. Hempel also found no Ambrosia at these sites in 2008 and 2009. However, in 2015 we were informed by Robyn Cobb, USFWS Corpus Christi Field Office, that Ambrosia was found in a different part of the Bishop City Park than we had previously searched; we surveyed this location for stem density and flowering stems in November 2015. In 2013, we were notified by Robyn Cobb that Alice Hempel had observed Ambrosia stems on private property in Kingsville, TX along the south side of General Cavazos Blvd between US 77 BR and Chandler. We were unable to obtain written permission of the landowner to survey this population after several attempts. Although some stems are in the road right of way, the traffic on the road made it too dangerous to survey. Taken altogether, these observations indicate that Ambrosia patches likely “wink in and out” in response to environmental conditions such as amount and/or timing of precipitation. Continued visits to sites where Ambrosia has been observed should be attempted. In addition, we observed invasive grass, mainly Kleberg Bluestem (*Dicanthium annulatum*), at all locations.

Annual Surveys and Fall Flowering Surveys

Polygon delineation: Using the USFWS criterion that all stems within 2 m of another stem are part of the same patch or polygon, we delineated two long and relatively thin polygons along Co Rd 73 in Robstown County Park and one large polygon in the Demonstration site at the park (Figure 5, Table 2). Hempel also noted two patches or “subpopulations” along the roadside (Hempel and Overath 2009). St. James Cemetery, which has the largest extent of Ambrosia outside NAS-K, had nine polygons of various shapes and sizes (Figure 3, Table 2). At the US 77 locations, we delineated two polygons (N and S) on the NB (east right of way) side and one smaller polygon on the SB (west right of way) side (Figure 6, Table 2). All polygons delineated at the Robstown sites in 2014 were slightly wider than in 2013 (Figure 5). The polygons at St. James cemetery were essentially the same; however, surveyors noted that several of the polygons could fragment in the future if insolated stems connecting the polygon disappeared. The US 77 sites could not be surveyed in 2014 due to access issues caused by the I-69 road construction; however, we observed plants in all three polygons.

Stem Survey results: Annual and fall flowering surveys revealed some interesting trends in stem density in and among the survey locations. During the study, the highest average densities occurred in the south polygon in the NB (east side) right of way on US 77 (64.3 stems per m^2 in November 2013; Figure 8) and Robstown County Park in polygon 2 (45.2 stems per m^2 in June 2014; Figure 9) and the Demonstration site (61.1 stems per m^2 in June 2014; Figure 9). The lowest densities were found the in the polygon on SB (west side right of way) US 77 location (4.3 stems per m^2 in November 2013; Figure 8) and polygon 1 in Robstown County Park (3.4 stems per m^2 in June 2014; Figure 9). The two polygons in Robstown Park are within a few meters of one another and must receive the same mowing regime.

Within site stem density tended to remain fairly constant or increased over time; however, the Robstown Co. Rd 73 polygons decreased in density between the June 2014 and November 2015 surveys (an order of magnitude for polygon 1 and 4-fold for polygon 2; Figure 9), while in polygon 2 at the St. James Cemetery density increased an order of magnitude (Figure 10). The main differences between these locations is that the polygons on the roadside at the park are mowed periodically; whereas the St. James Cemetery polygon is in an area that is not mowed and contains some trees and shrubs. Interestingly, precipitation in the months preceding the November 2015 survey was greater and more consistent than before the June 2014 surveys, but temperatures were greater, simply due to usual seasonal differences (Figure 7). Microsite differences may, therefore, be important for individual patch or polygon dynamics. Note, however, that there may also have been localized differences in rainfall between these sites. Unfortunately, NOAA climate data are not available for these years more locally.

Although we did not delineate polygons at Bishop City Park site in 2015, we did survey two adjacent patches (a mowed path or “road” and a grassy area). The “road” area had a higher average stem density (30.3 stems per m^2 with a standard deviation of 31.1) than the grassy area (12.5 stems per m^2 with a standard deviation of 14.1).

Flowering survey results: In 2013, we noticed an interesting pattern in percent flowering among highway/roadside sites. The US 77 polygons had an order of magnitude lower percent flowering than the Robstown Co. Rd. 73 polygons (Figure 11). In fact, the US 77 NB N polygon had no flowering stems and had obviously recently been mowed because surveyors notes seeing pieces of stems with inflorescences. Polygons 1 and 2 at Robstown County Park had not been mowed recently because grasses were over 0.5 m high and very dense. In 2015, no flowering stems were sampled in Robstown County Park polygon 1; however, 40% of stems were flowering in polygon 2 in the same location (Figure 12). There was no evidence of recent mowing and the polygons are adjacent; however, stem density had decreased drastically in polygon 1, and our survey missed flowering stems. At Bishop City Park, percent flowering in the “road” patch was ca. 2.5 times that of the grassy patch.

These results indicate that timing of mowing can have a large impact on flowering, and, if mowing takes places during the reproductive season, can cause total or at least high reproductive failure. However, the results from Robstown County and Bishop City Parks indicate that mowing alone is not necessarily detrimental to flowering if it is timed properly.

Curiously, we did not observe bur/seed production at any of these sites (or in management studies, see below) during the course of this study. Previous observations by Hempel the Robstown County Park Demonstration site indicate that burs of different stage may appear on the same plant as female inflorescences may mature and become receptive at different times (USFWS 2010). In addition, in recent observations in the greenhouse accessions at the San Antonio Botanical Gardens, the rare plant volunteer Shannon Smith found that achenes developed as soon as female flowers became mature and receptive (Smith, pers. comm.). Hempel also noted that the presences of burs/achenes in Fall 2009 after rains in September helped to break the drought, but did not indicate at which locations. Therefore, we expected to see burs during our flowering surveys.

We also examined the flowering phenology at these sites for 2013 and 2015. In 2013, flowering at the US 77 NB site appeared to be more advanced than the other sites as indicated by the majority (ca. 70%) of flowering stems being “female only” (only female flowers or flower buds observed, no evidence of male inflorescence because it was lacking or already had broken off), while “female only” stems comprised less than 30% of flowering stems at the other sites. Interestingly, the Robstown County Park Demonstration site had only one “male only” flowering stem while the roadside polygons nearby, which were surveyed on the same day, had “male only,” “female only,” and “both” stems, indicating that microsite as well as site may influence flowering phenology. We discuss additional issues relating to reproduction below after the Management Impacts section.

Physiological Ecology

We were unable to complete this part of the study due to problems with the LiCor 6400 Photosynthesis System. Details are discussed in the Significant Deviations section.

GPS/GIS

This work has not been completed. Details are discussed in the Significant Deviations section.

Soils

Sixteen soil characteristics varied over the 11 locations we sampled (Table 2); MANOVA results indicated that none of these varied significantly between areas with Ambrosia and without Ambrosia (Table 6). However, after correcting for multiple comparisons, 12 of these characteristics (% moisture, pH, % organic matter as estimated by the loss-on-ignition method, and nine elements) varied significantly among locations and no site X treatment interactions occurred (Table 6). Average % moisture was highest at St. James Cemetery (4.36% ± 0.38) and lowest at the NASK-Z2 site (2.24% ± 0.23). Soil pH was generally neutral with mean pH the most acidic at the Demonstration site (pH 6.69 ± 0.17) and the most basic at the NASK-Z1 site (7.58 ± 0.22). Average % organic matter (LOI) varied 2-fold from 3.57% (± 0.45) at the NASK-N1 site to 6.93% (± 0.71) at St. James Cemetery. No clear patterns or groupings were evident for these characteristics or for the nine elements from the Tukey’s multiple range tests (Table 2). For example, the NAS-K sites did not always group together and the Demonstration site grouped with different sites for different characteristics. The only obvious difference between the Demonstration site and the other locations was that the mean sodium concentration was 2- to 3-fold higher there (113 mg/kg ± 36.04) than the other locations, which did not differ statistically from each other. However, a more appropriate way to address this question might be a multiple-regression. Unfortunately, this type of analysis is not appropriate for our sampling design.

Interestingly, two of the three elements that did not differ among locations, phosphorus and potassium, are important macronutrients for plants; the third element that did not differ is boron (Table 6). Both the lack of differences among sites in macronutrients and the lack of consistent groupings among locations for those characteristics that do differ among locations suggest that taken together, these soil characteristics are unlikely to explain the differences in stem densities that we see in this study. Brannon et al. (1997) conducted a study of soil composition at two sites, St. James Cemetery and a site along US 77, in an attempt to explain differences in slender rush-pea population sizes at these locations. As in this study, they found no appreciable differences in phosphorus and potassium levels. Our results also indicate that Ambrosia may have a broader ecological amplitude and grow over a broader range of soil fertilities than is suggested by its current distribution. Whether it was a major component of throughout Texas coastal prairies or was restricted in range before European settlement of this area cannot be known.

Genetics

Microsatellite Markers

Three of the five primer pairs we genotyped were variable and two of those had more than 2 fragments in many individuals, indicating polysomic inheritance. Data from the variable loci, which were re-coded in light of this, yielded 22 different possible fragments or “loci” that were used in the analyses described above and focused on estimating clonal diversity. Based on a review of the transferability of microsatellite primers among species of varying degrees of taxonomic relationship (Barbará et al. 2007), we had expected to find about 40% (5-6) of the primers developed for the invasive *A. artemisiifolia*, common ragweed, would useful and variable in South Texas Ambrosia. We found 5 usable primer pairs but only three of them were variable. Additional optimization using other polymerases with different activity yielded no other useful markers.

The indications of polysomic inheritance we found in our data set should not be surprising. Polyploidy in known across the family Asteraceae, and South Texas Ambrosia has double the number of chromosomes (n = 36) that common ragweed (n = 18) has (Payne et al. 1964). Another member of the genus, *A. dumosa*, is not only polyploid but also varies in ploidy level across its range (Raven et al. 1968). Even with these challenges, we were able to obtain genetic information about Ambrosia using microsatellite markers that will be useful for managers; however, given the fact that our analysis is based on only three primer pairs, our conclusions also should be viewed with caution.

Overall diversity and clonal diversity and distribution: Analysis of the complete data set identified 31 clones (numbered 1-31) among the 136 stems successfully genotyped without any missing loci (Table 6). Thirteen of the 31 clones (42%) were only found once and 22 (71%) were found in only one location. Thirty-seven stems

(27%) were identified as clone 9, the most frequent clone (Table 6). This same clone was also the most widespread, occurring in seven of the 15 sampled areas (Table 6). It was the only clone found in the small patch sampled at St. James Cemetery. Two other clones (10 and 13) were almost as widespread, occurring in six areas each. The fact that the most wide-spread clone was also found at a site about 25 km away, indicates that at least some of the genotypes found at NAS-K would also be found on adjacent properties and other locations. However, the high number of unique clones (13 out of 31 found only once in the data set) also indicates that there may be some unique genotypes at NAS-K. This result also justifies our focus on sampling more areas less heavily rather than fewer areas more heavily, because we would have missed these unique clones.

Within the two most heavily sampled areas (11 and 14), individuals of the same clone tended to clump together, but not exclusively, especially for widespread clones (e.g. clones 9 and 10 in area 11 and clone 10 in area 14; Figure 15). Graphical examination of the distribution of clones of these areas (Figure 15), indicates that small patches of stems likely represent single clones. However, larger patches may include several interdigitating clones. Results for the congener *A. pumila* are somewhat similar in that at least in some cases 0.25 m^2 plots were dominated by a single clone, but clones were mixed in others. There were no obvious differences between the amount of clumping in these two areas even though they receive very different mowing regimes.

Genotypic (clone) diversity within and among areas: We examined three estimates of genotype or clone diversity within the 15 sampled areas. The number of genotypes or clones ranged from 1 to 10 (Table 7). Note that the two areas with 10 clones had large sample sizes; however, one of the areas with only one clone (StJ) also had a large number of samples. A better measure to compare among locations when sample sizes vary is the effective number of genotypes, which ranged from 1 to 5.90 (Table 7). This measure takes both sample size and eveness into account (Meirmans and Van Tienderen 2004). Genotypic diversity, an estimate of the probability that two random samples from a location will be the same clone or genotype, ranged from 0 for areas with only one clone to 1.00 for several areas in which each individual was a different genotype (Table 7). The overall genotypic diversity was 0.92 with 31% of the variation distributed across locations, which may indicate that sexual reproduction does occur in this species or that it occurred in the relatively recent past.

A similar study of an endangered congener in California, *A. pumila*, found the same number of clones but among more than 200 samples (McGlaughlin and Friar 2007). This species has the same number of chromosomes as our Ambrosia (Payne et al. 1964); therefore, differences in diversity cannot be accounted for by differences in ploidy. However, that study also sampled only nine plots and clones were never found in more than one plot, while this study focused on identifying as many clones as possible and a proportion of our clones were distributed across NAS-K. Genotypic diversity was much lower in this congener than in our Ambrosia, but is difficult to compare directly because McGlaughlin and Friar (1979) used a different index not suitable for our data because it is highly biased by differences in sample size (Meirmans and Van Tienderen 2004). Consequently, we judge that levels of clone diversity may be fairly similar in the two species, but the distribution of clones differs.

We also examined the probability of differences in clone or genotype frequencies between pairs of areas in a permutation test (Table 8). Because we had 105 comparisons, Bonferoni correction reduced our threshold P-value to 0.0005. Even with a high proportion of unique clones, we found little significant difference in genotypic diversity between pairs of areas (Table 8). The only pairs of areas that differed in genotype or clone frequencies were St James Cemetery and nine of the areas on NAS-K (Table 8). This finding may be explained by the fact that the wide-spread clones tended to be found in the same locations (e.g., clones 9 and 13 are both found in areas 7, 9, 11, 12, 14, and 20; Table 6), making clonal composition similar across areas. In addition, the small sample size for some areas, may have reduced our power to detect biologically important differences. Importantly, because we found no significant differences between pairs of locations from different land use types (e.g., areas 11 and 14) that receive different management practices such as differences in mowing intensity, it appears that such differences do not affect clone diversity within an area. None of the sites also used in the mowing study (see below) differed from one another in clone frequency, indicating that mowing regime may not increase the size of clonal stands as suggested by USFWS (2010).

Clone relationships: Another issue relating to clonal diversity is how similar or dissimilar clones are (e.g., highly similar clones may actually be parts of the same clone that have experienced different somatic mutations (Meirmans and Van Tienderen 2004)). The coding of our data as presence/absence of fragments due to indications of polysomic inheritance makes it difficult to directly measure clone relationships. However, the Euclidean distance or dissimilarity measure of Huff et al. (1993) can be used to assess differences among clones based on the number of fragments not shared between pairs of clones. Examining the UPGMA phenogram of Euclidean distances indicates that all clones group into one of six clusters that differ from one another by only 4-5 of 22 possible fragments (Figure 16). Within each of these clusters are clusters or pairs that differ by only 1 or 2 fragments. For example, clones 9 and 10, 12 and 19, 26 and 31, and 5 and 7 each differ from the other only by the presence/absence of one fragment. Such clones may represent cases in which somatic mutation of one clone produced the other, in which case they should really be considered the same clone (Meirmans and Van Tienderen 2004). If so, the number of clones in this study would decrease from 31 to 26. In addition, clones 9 and 10 are two of the most widespread clones. If they are actually the same clone, they would comprise 52 of 136 (38%) of the stems genotyped. The fact that the highest Euclidean distance (E) was just under E = 7 may indicate that little sexual reproduction is currently occurring and that all clones are highly related. For comparison, consider that in a study of a sexual reproducing grass, that is also capable of vegetative spread, within location distance ranged from E = 6 or 7 to E = 20 or 21 (Huff et al. 1993). In other words, our highest distance was on par with the lowest distance in that study. Another implication of the low dissimilarity among our clones is that the high genotypic diversity we saw may be a consequence of counting many highly similar clones as different genotypes and that effectively genotypic diversity is lower than it appears in this study. These caveats based on clone relationship as well as the low number of markers (3) used in our study mean that our conclusions must be taken cautiously. Genomic sequencing results, which will be based on hundreds of SNP markers, should give us a much clearer picture of the amounts of genetic diversity and how a management practice such as mowing may impact it.

Genomic sequencing

Awaiting results. See Significant Deviation section for details.

Management Impacts

Effects of Prescribed Burning

Plant reemergence: The fire cleared almost all vegetation in and around the burn plots (Figure 17B). Some small patches of grass remained but were completely brown. Interestingly, most Ambrosia stems were not consumed by the fire but were only dried and dead. Grass bunches that remained started to sprout new green shoots within one week of the fire. However, new Ambrosia stems did not reemerge until three weeks post-burn. The first stems we saw emerging occurred at the base of the dried stems left by the fire (Figure18). We saw no evidence of seedling emergence. Plots had mainly refilled with new, green growth by the first post-burn census (September 2013; Figure 17C) and looked indistinguishable from pre-burn plot within one year (August 2014; Figure 17A and D).

Ambrosia response to prescribed burning: The average number of stems per plot in the unburned area was greater than that in the burned area before the fire (11.8 ± 4 vs. 8.2 ± 1.9 stems per 0.25^2 plot) and remained so for most of the survey times throughout the study (Figure 19). Therefore, we focused on change in stem number post-burn (Figure 20). Stem density in the burn plots recovered quickly after the fire as the change in the average number of stems per plot was small (mean change varied from -1 in September 2013 to 2.2 in November 2013); by the May 2014 census the average number of had almost doubled, but then decreased somewhat (ca. 25%) by the August 2014 census (Figure 20). We also collected x-y coordinates for each Ambrosia stem at each census; however, lack of precision in the measurements made these data difficult to analyze. However, examination of these coordinates as well as photographs taken for percent cover estimates indicate that many of the stems that emerged in the initial period after the fire in the burn plots were likely re-emergence of the stems that were above ground before the fire (data not shown). The unburned plots had more substantial increases stem density after the burn for all census dates except September 2013, where the increase was minimal and January 2014, where there was a slight decrease compared to the pre-burn numbers (Figure 20). Differences in the change in stem density over time as well as between the burned and unburned plots were significant ($P_{time} < 0.001$; $P_{treatment} = 0.002$); however, this difference was not in the direction expected as the unburned plots added more stems than the burned plots for most census dates, indicating that Ambrosia did not benefit from the fire, but was not harmed either.

Other response variables tell a somewhat different story. We measured plant height at the May and August 2014 censuses. Overall, mean plant height did not differ between the burned and unburned plots ($P_{treatment} = 0.265$; Figure 21). However, plant height in both areas increased significantly between the two census dates ($P_{time} < 0.001$; Figure 21) with a significantly greater mean increase (ca. 35%) in the burn plots than in the unburned plots (ca. 27%), as indicated by the significant interaction term ($P_{intereaction} = 0.001$). Percent flowering also indicated some potential benefit of prescribed burning. In 2013, mean percent flowering stems in the burn plots was ca.

11% higher than in the unburned plots, a marginally insignificant difference ($P = 0.6$; Figure 22), while in 2015 mean percent flowering was the burned areas lower than in 2013, but significantly greater, almost double, than in the unburned areas ($P = 0.01$). Because Ambrosia is protandrous, male inflorescences open first, we also examined differences in flowering phenology between the two areas. There is some evidence of differences in flowering phenology in both years (Figure 23). In 2013, the unburned plots still had some plants that were "male only" with no evidence of female flowers forming yet, indicating that at least some stems in the unburned plots were "behind" those in the burned plots. However, in 2015 the unburned area about 30% female only flowers with no evidence of male inflorescences, which may have already withered and broken off, indicating at least some stems in this area were "ahead" of those in the burned area, which had no "female only" stems. (Note that these numbers could not be examined statistically.) We should also note that we saw no evidence of bur production in either year at this site. In 2013, we also censused the plots in January and observed no burs. These observations, along with the fact that we did not observe seedling emergence in the burned plots, indicate that at least at this time, sexual reproduction is not effectively contributing to the population at this site.

We also examined percent cover of Ambrosia as a proxy of plant size, reasoning that perhaps prescribed burning would promote larger plants that occupied more space. Before the fire, mean percent Ambrosia cover in the unburned plots was almost twice that in the burned plots and remained higher in the unburned plots across the study (Table 9); therefore, we focused on the change in percent cover after the fire. Change in mean percent cover was 2.5 times larger in the unburned plots in September 2013 and essentially the same in August 2014 (differences marginally not statistically different, $P_{treatment} = 0.08$; Table 10). Changes in percent cover, overall, did not differ statistically between the two census times either ($P_{time} = 0.13$). Thus, we have no hard evidence that the Ambrosia stems in the burned areas were lusher or larger than those in the unburned area.

Response of other vegetation to prescribed burn: Due to the lack of obvious response of Ambrosia stem density or percent cover to the prescribed burn, we also examined the percent cover data to verify that the fire had the desired effect of suppressing grass cover, which was mainly the invasive Kleberg bluestem. Mean change in percent cover of grasses decreased in both the burned and unburned plots in the first census after the fire, but the decrease in the burn plots was double that in the unburned plots (Table 10). By the August 2014 census, the mean percent grass cover had increased again (Table 9), but the grass cover was still less than before the fire in burn areas. These differences were statistically significant ($P_{treatment} = 0.027$; $P_{time} < 0.001$), indicating that the fire did suppress invasive grasses, but that this effect decreased over time.

Mean percent forb cover also increased after the fire (Table 9, Table 10; $P_{time} = 0.002$), but increased similarly in both the burned and unburned plots ($P_{treatment} = 0.405$). Interestingly, we noticed in a drastic change in cover for the native species *Menodora heterophylla* in one plot in the burn area. While this species was present in both the burn and the unburned areas before the fire, it was the main forb species in burn plot 4 in the September 2013 census, covering ca. 75% of the plot (data not shown). By the August 2014 census, however, grasses had increased dramatically again in this plot and the cover of *M. heterophylla* was less than 20%. We examined the percent cover data for other instances of dramatic response by individual species, but did not observe any.

The major drawback of this study is that there are differences between the burned area and the area that we used as an unburned control. For example, the unburned area has trees and shrubs and the burned area does not. In addition, grasses (mainly Kleberg bluestem) were more abundant in the burned area before the fire (Table 9). These differences, as well as large plot-to-plot differences in all response variables, may have limited our ability to detect real biological responses. What our study does indicate is that Ambrosia is fire adapted, as might be expected for a prairie species. Ambrosia stems were killed but not consumed by the fire, in contrast to most of the other vegetation, and, importantly, most stems re-sprouted within a few weeks, and stem density and percent cover were close to pre-burn levels within 5-6 weeks of the fire. These results are similar to effects of fire on Ambrosia noted by other observers (USFWS 2010). In addition, we have some suggestion that burning may encourage sexual reproduction (at least flowering); however, the lack of seedling emergence after the fire and the lack of mature burs/achenes during the study period calls into question the significance of this result.

The prescribed burn was effective at reducing grass cover, at least initially, which is in agreement with other studies of the effects of fire on invasive Old World Bluestems. Simmons et al. (2007) reported that growing season fire (i.e., summer) reduced the abundance of King Ranch Bluestem (*Bothriochloa ischaemum*) for a year in central Texas. In the Texas coastal prairie, studies at the Welder Wildlife Refuge indicate that native species benefit from growing season fire during drought, and King Ranch bluestem can at least be kept from increasing by (e.g., Twidell et al 2012). In addition, the response of *M. heterophylla* suggests that other native species that co-occur with Ambrosia have the potential to benefit from prescribed burns, at least in particular microclimates, and, therefore, fire may be an effective management tool in prairie restoration.

Effects of Mowing

This study took advantage of mowing regimes already in place at NAS-K. Although the study itself is short-term, the main mowing regimes (not mowed, tractor mowed, and zero-turn mowed) have been in place for some time; therefore, unlike in the prescribed burn study described above, we have no “before and after” comparison to make here.

Vegetative response of Ambrosia to mowing: Stem density (average number of stems per 0.25m^2 plot) varied within and among mowing regimes at the July census with mean stem density under the not mowed regime about 67% of that under any kind of mowing in June 2014; however, this difference was not significant (P = 0.84). At the Nov/Dec 2014 census, mean number of stems was 70-90% of the means for July and did not differ statistically across the mowing regime (Figure 24).

In July 2015, only three of the transects could re-located. For all the tractor mowed sites, one of the zero-turn mowed sites (Z2), and one of the not mowed sites (N2), the lawn staples or wooden stakes that marked the transect ends were no longer evident. We attempted to locate individual plot corners, so that we could measure from there to the transects but that approach was not successful. In the three sites where transects and plots were relocated, the Ambrosia populations had crashed. At not mowed site N1, only two of the plots still contained stems (6 stems each). The other three plots were essential bare ground; however, what caused this to happen is unclear as there was no obvious sign of fire or other disturbance such as tire tracks. Note that during the fall flowering survey in November 2015, the N1 site had no stems remaining. The other two sites, Z1 and Z3, had no Ambrosia stems in the plots or nearby in July 2015, but one stem was found at the Z3 site and the Z1 site still had no stems in November. Note that for these zero-turn mowed sites, mowing height appeared to be much shorter than the target height (see discussion below). We noticed the presence of *Parthenium hysterophorus* in all sites during this period, where it seemed most prevalent in the not mowed and tractor mowed sites. (Interestingly, it was also quite common in our neighborhood in Corpus Christi, TX this summer and fall.) Lehman et al. (2005) call this species "a weed pest." Perhaps its potential interactions with Ambrosia and other natives should be investigated if it continues to be prevalent at NAS-K in the future.

We also recorded plant height as a measure of plant performance. Unsurprisingly, at the July 2014 census, Ambrosia stems under the not mowed regime were 30% taller than stems under tractor mowing and more than 2.5 times the size of stems under the zero-turn mowing; however, these differences were not statistically significant (P = 0.123). These differences were accentuated at the Nov/Dec 2014 census; stems from not mowed sites more than twice the height of stems at the tractor mowed sites and 3.5 times the height of those at the zero-turn mowed sites (P = 0.061). However, even using a nested design to account for environmental heterogeneity, these differences were marginally not statistically significant. By chance, some of our plots at the tractor mowed sites were located in slight depressions. Due to the width of the mowing device, plants located in these depressions were taller than surrounding plants. These taller plants located in slight depressions added to the heterogeneity and may be a reason why plant height among mowing regimes was not statistically significant or marginally significant. It seemed to the surveyors that stem density was higher in these slight depressions and taller plants in these slight depressions were in better condition than surrounding shorter plants. However, we cannot attribute apparent differences in stem density or plant condition to differences in effective mowing height because differences may in part be due to plants in the slight depressions having better access to water and nutrients. We think it important to note that for mowed locations, Ambrosia mean height was much lower than the target heights we were given (Table 4). In particular, the mean height for Ambrosia at Z1, which experienced a complete collapse by July 2015 and still had no stems in November 2015, was ca. 3 cm in both the 2014 censuses. It is highly likely that this level of mowing has a detrimental cumulative effect on Ambrosia.

Average number of branches per stem per plot was another growth proxy we examined, thinking that mowing might cause stems to branch more. While we did note some highly branched individuals (> 5 branches per stem), these were rare. Mean number of branches was very slightly higher under the tractor mowing regime in July 2014 than the other two mowing regimes (Figure 26), but this difference were marginally not statistically significant (P = 0.088). In Nov/Dec 2014, the mean number of branches per stem were not statistically different among the mowing regimes (P = 0.177).

Percent cover of Ambrosia and other species: Percent cover of Ambrosia was estimated for the July 2014 census. Ambrosia cover varied two- to three- fold among the not mowed and zero-turn mowed sites (Table 11), but overall was almost twice as high under the not mowed and tractor mowed regimes than under zero-turn mowing. However, these differences were not statistically significant (P = 0.468), likely due to the heterogeneity among sites of a mowing regime. Similarly, although mean percent grass cover appears to be ca. 1.5 times higher without mowing, and mean percent forb cover is 2.5-3 times higher under the tractor mowing regime, these differences are not statistically significant (P = 0.785 and P = 0.192, respectively). Closer examination of the data indicates that mean percent cover varies quite a bit within mowing regimes; this level of heterogeneity is likely masking biological differences among mowing regimes. Note that all of these measures focus on what Ambrosia is doing vegetatively above ground. As mentioned above, our microsatellite markers studies also indicate that mowing regime may not increase clonal spread of Ambrosia belowground.

Effects of mowing on Ambrosia reproduction: We surveyed for flowering stems in November/December 2014 in the plots established in July 2014. Mean percent flowering stems per plot was two orders of magnitudes higher under no mowing than under tractor mowing and no stems were flowering in the zero-turn sites (Table 12). One of the not mowed sites (N3) had been mowed in the fall, so we dropped it from the study. We also were not able to relocate the transect or any of the plots at the T3 site. Our survey was also hampered by inclement weather and availability of personnel and took several weeks to complete. Therefore, we are cautious about interpreting these data and took the opportunity to collect more data in November 2015. In 2015 some sites still had no stems and one site (Z3) had only one stem that was sampled, which happened to be flowering (Table 12). Ignoring these sites, flowering was higher in the not mowed site N2 (20%) than any of the tractor mowed sites (2-15%) and the one zero-turn mowed site that had a reasonable number of stems (14%). Lack of replication of and many zeros in the data set made statistical analysis invalid, but these data show a trend higher percent flowering stems in not mowed sites that supports the trend seen in 2014.

We also examined flowering phenology in both years. In 2014 only three sites had flowering stems during our surveys. The one tractor site had one flowering stem that was “male only” (Figure 27). The two not mowed sites differed from one another with the N1 having a much higher proportion of “both” stems and N2 having a relatively even spread of the three inflorescence types. In 2015 low or numbers of flowering stems at some sites make interpretation difficult, but it appears that the not mowed site may be farther along in flowering as it has some “female only” stems. As with our burn study and the fall flowering surveys in other sites, we observed no burs in any of our plots in either year.

Overall Impacts of Management Practices

Both burning and mowing have been used in an attempt to control invasive grasses that constitute a major threat to Ambrosia population viability (USFWS 2010); therefore, understanding their impacts on Ambrosia biology is important. Based on our studies here, fire appears to have few detrimental effects on Ambrosia as stems resprout rapidly and return to pre-burn densities within a month or two. In addition, fire may increase percent of flowering stems, but the importance of sexual reproduction in this species is still unclear. However, we should point out that the prescribed burn in our study was carried out after a drought had broken and the total precipitation in September 2013 was much greater than in 2012 (Figure 7). Whether Ambrosia will respond as well during a drought or with less precipitation after a prescribed burn is an important question as some evidence suggests that invasive bluestems might be best controlled by timing fire during the growing season during a drought (Havill 2015).

Mowing also appears to not have large detrimental effects on vegetative growth compared to no mowing, although a benefit from controlling grass competitors also was not obvious in our study contrary to other mowing studies at NAS-K (Bush et al 1994 and Garvon 2005). We are concerned by the population crashes in two of the zero-turn mowed sites. In particular, we suspect that mowing to 3 cm in one of the sites contributed to its demise. Therefore, the height of mowing needs to be close enough to suppress competitors but not so low that Ambrosia plants are too short. Fall flowering surveys in all sites as well as in this mowing impact study suggest that timing of mowing *is* important and should be avoided during flowering. However, as stated elsewhere, how important sexual reproduction is currently in these sites is unclear.

Additional Reproduction Observations

As noted elsewhere, we have not observed bur/achene formation or seedlings during this study. We had hoped to perform some controlled crosses in Fall 2014, but difficulties with completing the November/December survey of the mowing sites at NAS-K kept us occupied in late fall. By the time we completed that survey and had time to get back into the field, flowering had completed. However, Shannon Smith, the rare plants volunteer at the San Antonio Botanical Garden (SABot), who has shared his studies and observations on the greenhouse accessions. Mr. Smith first pointed out that the records for their accessions are unclear and contradictory. He believes that they may be growing only one clone from one accession that was collected in the Sinton, TX area. However, we are not aware of any instance of Ambrosia from that location in any of the records. The number of accessions is important because self-incompatibility, albeit a form that is “leaky,” allowing some self-seed formation, has been well-documented in *Ambrosia artemisiifolia* (Friedman and Barrett 2008). Friedman and Barrett (2008) noted that isolated plants set few seeds. If South Texas Ambrosia has a similar form of self-incompatibility, then crossing within a clone may also result in the production of few seeds. Mr. Smith has noted that greenhouse plants release pollen before female flowers become receptive, as expected in a protandrous species. His description of pollen dropping from male inflorescences onto female flowers, which occur in the leaf axils below the male inflorescence, sounds very much like that described by Friedman and Barrett for *A. artemisiifolia*. On plants in female flowers opened and became receptive, fruits formed. However, none of several brown and dry fruits (burs) he examined had an achene. Hempel (cited in USFWS 2010) found a “substantial number” of empty burs in the Demonstration site at Robstown County Park in Fall 2007. These observations are suggestive of at least partial self-incompatibility in South Texas Ambrosia. Further studies are needed to confirm this, but they should be augmented with genotyping to ensure that at least some crosses are conducted between different clones.

Acknowledgements

We gratefully acknowledge the support of the Texas Parks and Wildlife Department through Section 6 Grant Contract # 415607. PCR optimization and microsatellite analysis of samples from the Naval Air Station—Kingsville (NAS-K) were funded through contract N69450-10-M-4898 from the US Navy. We thank Alin González for assistance with both field and lab work and, especially GIS and map preparation. Members of the Overath Population Genetics Lab at Texas A&M University—Corpus Christi, especially Brandi Kutil, PhD, Grayce Palmer, and Cori Speights provided assistance in the lab and Phil Jose provided assistance in collection of genetic samples from NAS-K. We also acknowledge the assistance of members of the TAMU-CC Islander SEEDS chapter for recording flowering data in the 2013 surveys. We thank Robyn Cobb and Pat Clements of the USFWS Corpus Christi Field Office for helpful discussions and assistance throughout this work. We appreciate Sandra Rideout-Hanzak, PhD including the Ambrosia area in her controlled burn of slender rush-pea at St. James Cemetery, which allowed us to study the effects of burning. We are grateful for advice Millie Burrell, PhD provided concerning genomics approaches and microsatellites and the assistance of Daisy Zhang, PhD for discussions concerning PCR optimization. We thank the Geospatial Sciences Lab at the Harte Research Institute for Gulf of Mexico Studies at TAMU-CC for periodic use of a Trimble. We also thank Shannon Smith, Rare Plant Volunteer, San Antonio Botanical Garden (SABot) for sharing his observations of Ambrosia reproduction and information about greenhouse accessions.

This work was conducted under Federal Permit TE25736A and State Permit SPR-0812-960 to RDO. We gratefully acknowledge the permission of St. James Parish for access to their cemetery. We also thank the US Navy for permission to conduct the mowing study at the Naval Air Station-Kingsville and especially thank Jeremy Adams, Natural Resources Manager, for assistance accessing the sites, and Ken Mahaffey for assistance in choosing sites. We acknowledge the permission of Nueces County and the City of Bishop for allowing us to perform surveys at Robstown County Park and Bishop City Park, respectively.

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Table 1. Locations visited and surveyed during this study 2012-2015. Condition in 2009 based on observations by Dr. Alice Hempel as reported in Hempel and Overath's 2009 interim report. (Map number refers to the number code on the map in Figure 1).

Site (map #)	Condition in 2009	2012	2013	2014	2015
Bishop City Park, Nueces Co (1)	Extirpated/Unknown	No plants found	Not Visited	Not Visited	Fall Flowering Survey
Robstown Park, Nueces Co., Co Rd 73 (2)	Extant	No plants found	Summer Annual Survey Fall Flowering Survey	Summer Annual Survey	Fall Flowering Survey
Robstown Park, Nueces Co., Demonstration Site (3)	Extant	Plant found	Summer Annual Survey Fall Flowering Survey	Summer Annual Survey	No Surveys
St. James Cemetery, Near Bishop, TX Nueces Co. (4)	Extant	Plants found	Summer Annual Survey, Began Burn Study, including fall flowering	Summer Annual Survey, Continued Burn study monitoring	Fall Flowering Survey
Naval Air Station—Kingsville, TX Kleberg Co. (5)	Extant	Lab members participated in USFWS surveys, collected samples for genetic studies	Not Visited	Began Mowing Study	Continued Mowing Study, Fall Flower Survey
Private Property Kingsville, TX Kleberg Co. S side of General Cavazos btw US 77BR and Chandler (6)	N/A	N/A	Plants found	Not Visited	Not Visited

Table 1 con’t

Site	2009 Condition	2012	2013	2014	2015
US 77 Northbound Right of Way (west side of highway), Nueces Co. (7)	Extant	Plants found	Summer Annual Survey Fall Flowering Survey	No Surveys	Not Visited
US 77 Southbound Right of Way (east side of highway), Nueces Co. Nueces-Kleberg Co. Line (8)	Extant	No plants found	Summer Annual Survey Fall Flowering Survey	No Surveys	Not Visited
Violet, TX (RR tracks along Hwy 44, ½ mi. west of Violet) Nueces Co.	Extirpated	No plants found	Not Visited	Not Visited	Not Visited

Table 2. Polygon and transects information for locations first censused during annual and Fall reproductive surveys in 2013.

Location	**Polygon**	**Transect Length (m)**	**Number of quadrats sampled**	**GPS coordinates** (Transect 0-m mark)
Robstown County Park	1	20	10	N 27 48.920 W 97 40.913
	2	37	10	N 27 48.001 W 97 40.899
	Demo[a]	32	10	N 27 48.905 W 97 40.742
St. James Cemetery	1	27	10	N 27 34.166 W 97 47.626
	2	50	10	N 27 34.146 W 97 47.623
	3	37	10	N 27 34.143 W 97 47.618
	4	10	5	N 27 34.121 W 97 47.642
	5	7	5	N 27 34.115 W 97 47.647
	6	10	5	N 27 34.116 W 97 47.654
	7	NA	Total stem count	N 27 34.149 W 97 47.634
	8	NA	Total stem count	N 27 34.151 W 97 47.636
	9	6	5	N 27 34.146 W 97 47.648
US 77 NB	N	37	10	N 27 33.453 W 97 48.082
	S	22	10	N 27 33.442 W 97 48.098
US 77 SB		13	10	N 27 33.654 W 97 48.855

[a] Demo = Demonstration site

Table 3. Mean and standard deviation (s.d.) for 16 soil characteristics for samples collected from 11 sites in areas with Ambrosia (Amb) and without Ambrosia (NoAmb). Units for all elements are mg/kg.

Site[a]	Area		% Moist	pH	LOI[b]	Aluminum	Boron	Barium	Calcium	Iron	Potassium
Demo	**Amb**	mean	3.35 BC	6.74 C	3.42 BC	11.73 A	0.86	30.91 D	3792.11 C	0.32 AB	770.73 A
		s.d.	0.13	0.21	0.07	1.16	0.02	0.63	438.30	0.04	56.84
	NoAmb	mean	3.26	6.64	4.17	9.93	0.74	31.37	3524.07	0.32	676.49
		s.d.	0.05	0.19	0.20	1.90	0.14	2.07	504.31	0.00	42.80
RobP	**Amb**	mean	3.24 B	7.16 ABC	6.75 AB	2.15 BC	1.21	52.81 AB	32637.54 AB	0.37 A	597.18 AB
		s.d.	0.13	0.14	1.54	1.75	0.25	1.49	5123.51	0.06	46.44
	NoAmb	mean	3.59	7.08	5.41	4.85	0.83	39.37	12876.40	0.39	611.76
		s.d.	0.10	0.08	0.42	2.84	0.01	4.15	4340.43	0.02	10.37
StJC	**Amb**	mean	4.13 A	7.11 BC	6.66 A	3.18 B	1.52	55.63 A	29659.61 AB	0.23 BCD	674.30 AB
		s.d.	0.17	0.15	0.73	2.12	0.46	1.46	11370.66	0.03	54.65
	NoAmb	mean	4.81	6.66	7.48	4.58	1.20	53.71	11636.61	0.25	680.43
		s.d.	0.06	0.57	0.12	1.02	0.66	7.72	6387.78	0.04	25.51
N1	**Amb**	mean	2.37 DE	7.38 AB	3.62 C	1.53 BC	0.97	52.12 AB	38939.36 A	0.21 CD	541.12 B
		s.d.	0.34	0.10	0.02	0.04	0.41	0.72	648.02	0.04	85.94
	NoAmb	mean	2.36	7.30	3.53	2.57	0.80	53.09	40183.37	0.21	547.74
		s.d.	0.21	0.28	0.55	2.26	0.18	1.26	1513.08	0.01	58.91
N2	**Amb**	mean	3.04 BC	7.29 AB	5.45 ABC	0.70 C	0.94	54.31 AB	41458.75 A	0.17 D	575.08 AB
		s.d.	0.49	0.04	1.84	0.19	0.47	3.57	1643.62	0.02	125.18
	NoAmb	mean	3.16	7.22	5.75	0.00	1.12	48.74	41356.89	0.13	581.06
		s.d.	0.07	0.19	0.47	0.00	0.84	7.66	2826.21	0.02	137.34

[a] Site codes: RobP = Robstown Park, Demo = Demonstration Site at RobP, StJC = Saint James Cemetery, other site codes refer to areas in the mowing study at the Naval Air Station—Kingsville: N1 and N2 are not mowed sites. T1, T2, and T3 are tractor mowed sites and Z1, Z2, and Z3 are zero-turn mowed sites. [b] LOI = % organic matter by loss on ignition.

Site[a]	Area		% Moist	pH	LOI	Aluminum	Boron	Barium	Calcium	Iron	Potassium
T1	**Amb**	mean	2.88 CDE	7.36 AB	6.08 ABC	3.04 BC	1.42	33.48 D	12419.22 BC	0.22 BCD	680.25 AB
		s.d.	0.17	0.01	0.27	0.93	0.03	4.16	1732.58	0.04	1.41
	NoAmb	mean	2.46	7.21	5.42	2.59	1.55	29.17	11004.99	0.22	710.55
		s.d.	0.05	0.43	1.02	0.18	0.03	2.65	4108.04	0.04	8.07
T2	**Amb**	mean	2.77 BCD	7.00 ABC	6.60 ABC	3.47 BC	1.41	28.06 D	10094.86 BC	0.24 BCD	720.15 AB
		s.d.	0.46	0.13	1.47	0.95	0.06	1.16	6161.65	0.01	139.47
	NoAmb	mean	3.06	7.33	5.50	2.88	1.48	39.03	18783.02	0.23	657.41
		s.d.	0.22	0.05	0.15	0.08	0.11	4.06	5913.43	0.00	54.68
T3	**Amb**	mean	3.00 BCD	7.43 AB	6.01 ABC	3.01 BC	1.24	36.80 CD	11113.04 BC	0.19 CD	582.60 AB
		s.d.	0.04	0.04	0.47	0.27	0.01	6.72	6403.48	0.01	26.17
	NoAmb	mean	2.90	7.29	5.24	2.93	1.29	38.18	14105.45	0.19	633.38
		s.d.	0.10	0.17	0.43	0.28	0.09	3.29	3964.81	0.01	14.89
Z1	**Amb**	mean	2.81 BCDE	7.71 A	6.90 A	1.60 BC	1.61	49.90 ABC	27581.38 AB	0.24 BC	659.04 AB
		s.d.	0.06	0.06	0.30	0.88	0.25	14.50	21301.48	0.05	19.99
	NoAmb	mean	2.79	7.40	6.61	1.64	1.17	45.68	25874.99	0.28	557.68
		s.d.	0.93	0.20	3.69	0.88	0.74	9.13	20992.39	0.12	56.12
Z2	**Amb**	mean	2.36 E	7.51 A	4.60 ABC	2.80 BC	1.42	32.47 D	15514.85 BC	0.22 CD	553.62 B
		s.d.	0.12	0.05	1.03	0.57	0.49	0.35	119.94	0.02	88.18
	NoAmb	mean	2.11	7.36	5.10	1.45	1.63	29.54	11144.71	0.21	549.96
		s.d.	0.29	0.03	0.01	0.91	0.01	1.03	589.68	0.02	101.85
Z3	**Amb**	mean	2.58 DE	7.25 A	4.77 ABC	4.71 BC	1.02	39.16 BCD	23443.31 AB	0.23 BC	585.83 AB
		s.d.	0.09	0.16	0.36	0.11	0.09	2.17	2047.60	0.02	18.87
	NoAmb	mean	2.11	7.71	4.23	2.41	0.77	43.51	33763.29	0.27	579.18
		s.d.	0.01	0.02	0.59	2.28	0.06	3.42	8053.09	0.03	75.57

Table 3 –Part 2

Site[a]	Area		Magnesium	Manganese	Sodium	Phosphorus	Sulfur	Silicon	Zinc
Demo	**Amb**	mean	637.77 A	3.96	93.43 A	3.73	11.22 C	94.77 A	0.07 B
		s.d.	61.21	0.05	31.92	3.97	7.26	2.62	0.02
	NoAmb	mean	664.06	8.40	134.29	3.53	11.01	88.13	0.16
		s.d.	9.83	4.91	34.78	1.43	7.10	4.05	0.00
RobP	**Amb**	mean	340.66 C	6.06	49.46 B	5.38	18.20 C	78.57 AB	0.30 B
		s.d.	19.75	1.41	4.60	3.21	3.84	1.23	0.10
	NoAmb	mean	278.44	5.21	34.50	3.92	8.25	92.21	0.17
		s.d.	19.36	1.50	0.93	1.83	3.35	6.82	0.04
StJC	**Amb**	mean	473.46 B	6.15	69.75 B	2.52	20.49 BC	83.70 A	0.16 B
		s.d.	25.31	3.19	11.92	0.78	13.30	9.95	0.08
	NoAmb	mean	482.54	2.63	54.88	2.19	7.91	98.85	0.10
		s.d.	84.24	0.16	3.71	1.21	2.62	1.02	0.00
N1	**Amb**	mean	427.03 BC	7.72	46.99 B	3.70	29.78 AB	55.63 D	0.14 B
		s.d.	6.00	2.86	2.12	0.07	3.69	3.12	0.05
	NoAmb	mean	392.64	7.37	42.24	2.62	32.61	56.92	0.10
		s.d.	39.81	0.98	2.94	1.16	4.89	2.64	0.02
N2	**Amb**	mean	418.55 B	5.15	67.95 B	4.20	29.43 A	59.54 CD	0.27 B
		s.d.	24.81	1.04	5.27	1.75	3.67	8.59	0.02
	NoAmb	mean	559.15	4.35	56.46	7.50	41.19	63.38	0.14
		s.d.	141.07	0.42	7.63	4.05	13.99	9.83	0.08

[a] Site codes: RobP = Robstown Park, Demo = Demonstration Site at RobP, StJC = Saint James Cemetery, other site codes refer to areas in the mowing study at the Naval Air Station—Kingsville: N1 and N2 are not mowed sites. T1, T2, and T3 are tractor-mowed sites and Z1, Z2, and Z3 are zero-turn-mowed sites. [b] LOI = % organic matter by loss on ignition.

Site[a]	**Area**		**Magnesium**	**Manganese**	**Sodium**	**Phosphorus**	**Sulfur**	**Silicon**	**Zinc**
T1	**Amb**	mean	351.92 C	5.36	64.38 B	3.76	14.40 BC	79.84 ABC	0.15 B
		s.d.	26.93	0.16	1.13	0.97	0.30	1.83	0.02
	NoAmb	mean	346.16	4.61	49.15	5.15	17.81	72.43	0.14
		s.d.	31.81	0.34	7.18	0.54	0.86	1.48	0.04
T2	**Amb**	mean	358.93 BC	5.11	63.44 B	4.57	15.77 BC	79.95 ABC	0.14 AB
		s.d.	114.75	0.98	3.02	2.31	1.62	9.60	0.05
	NoAmb	mean	362.04	4.39	58.09	4.03	16.59	76.01	0.58
		s.d.	34.04	0.31	3.39	0.87	1.30	2.41	0.48
T3	**Amb**	mean	326.62 C	4.29	40.00 B	2.96	8.59 C	73.24 ABCD	0.15 B
		s.d.	25.05	0.15	0.83	0.95	1.62	3.74	0.02
	NoAmb	mean	336.70	4.21	44.08	3.99	13.06	76.21	0.12
		s.d.	1.53	1.20	3.89	0.27	2.66	2.49	0.05
Z1	**Amb**	mean	378.49 BC	5.92	49.56 B	5.42	19.51 ABC	72.15 BCD	1.31 A
		s.d.	100.85	0.98	11.51	1.34	12.57	6.12	0.56
	NoAmb	mean	388.31	7.01	52.20	4.64	23.41	66.28	1.97
		s.d.	106.48	1.99	8.58	3.05	12.93	22.77	2.44
Z2	**Amb**	mean	321.16 C	5.40	52.70 B	4.37	16.87 BC	65.47 CD	0.84 AB
		s.d.	60.33	2.01	5.82	0.46	0.52	2.50	0.96
	NoAmb	mean	331.91	5.99	38.03	6.57	17.05	57.33	1.03
		s.d.	13.66	0.02	3.59	0.08	0.55	11.50	0.69
Z3	**Amb**	mean	352.96 BC	4.81	40.68 B	4.11	17.26 ABC	69.13 CD	0.25 B
		s.d.	0.89	0.51	3.77	1.52	2.64	3.13	0.03
	NoAmb	mean	348.64	6.87	47.42	3.64	21.27	56.00	0.22

		s.d.	41.53	0.58	5.01	1.17	4.13	6.96	0.03

Table 4. Mowing study locations at Naval Air Station—Kingsville and descriptions of mowing regimes.

Site	USFWS Area Number[a]	GPS Coordinates	Mowing Regime	Description of Mowing Regime[b]
N1	near area 17	N27 28.166 W97 49.716	Not mowed	Not mowed regularly
N2	11	N27 29.436 W97 49.450		
N3	9 (inside fence)	N27 29.221 W97 48.867		
T1	14	N27 29.566 W97 49.123	Tractor	Kept at 7-11 in height with tractor mower
T2	15	N27 29.529 W97 49.162		
T3	8	N27 29.426 W97 48.987		
Z1	6	N27 29.835 W97 49.207	Zero-turn	Kept at 4-7 in height with zero-turn mower
Z2	9 (outside fence)	N27 29.217 W97 48.873		
Z3	13	N27 29.595 W97 49.037		

[a] USFWS area numbers are from Garvon (2005).

[b] Mowed areas mowed “as needed” to maintain target height (LT Christopher Waldrop, CEC, USN, FEAD Director, NAS-K, pers. comm.). Note that the target heights are much taller than we were first advised (4 in and 2 in, respectively) by Mr. Ken Mahaffey, who assisted us in site selection.

Table 5. Summary table from MANOVA analysis of 16 soil characteristics followed by univariate ANOVA tables for individual elements.

Summary		DF	Pillai	Approx. F	Num df	Dem df	P
MANOVA	Site	10	6.5100	2.33263	160	200	8.185e-9
	Treatment	1	0.5915	0.99531	16	11	0.5171
	Site*Treatment	10	4.7143	1.11487	160	200	0.2325
	Residuals	26					

Element	Factor	DF	Sum Sq	Mean Sq	F	P
% Moisture	Site	10	0.0018575	1.8575e-04	26.043	4.754e-11
	Treatment	1	0.0000014	1.4120e-06	0.198	0.66000
	Site*Treatment	10	0.0001361	1.3608e-05	1.908	0.09042
	Residuals	26	0.0001854			
pH	Site	10	10 2.6601	0.266008	7.0348	3.032e-05
	Treatment	1	1 0.07709	0.077086	2.0386	0.16525
	Site*Treatment	10	10 0.7107	0.071067	1.8794	0.09547
	Residuals	26	26 0.9832	0.037813		
LOI	Site	10	0.0056414	0.00056414	4.5892	0.0008454
	Treatment	1	1 0.00006	0.00005760	0.4686	0.4996864
	Site*Treatment	10	10 0.0006	0.00006250	0.5084	0.8685672
	Residuals	26	26 0.0032	0.00012293		
Al	Site	10	287.525	10 28.7525	14.4205	3.186e-08
	Treatment	1	0.044	1 0.0440	0.0221	0.8830
	Site*Treatment	10	24.736	2.4736	1.2406	0.3127
	Residuals	26	51.841	1.9939		
B	Site	10	3.0497	0.304974	2.4174	0.03446
	Treatment	1	0.1401	0.140069	1.1103	0.30172
	Site*Treatment	10	0.5913 0	059129	0.4687	0.89510
	Residuals	26	3.2801	126158		
Ba	Site	10	3954.7	395.47	17.8041	3.36e-09
	Treatment	1	32.2	32.19	1.4493	0.23948
	Site*Treatment	10	432.1	43.21	1.9451	0.08424
	Residuals	26	577.5	22.21		
Ca	Site	10	57678318	576783179	9.0695	3.146e-06
	Treatment	1	10496962	104969622	1.6506	0.2102
	Site*Treatment	10	10653859	106538595	1.6752	0.1406
	Residuals	26	16534919	63595843		

Element	Factor	DF	Sum Sq	Mean Sq	F	P
Fe	Site	10	0.191631	0.0191631	12.2193	1.746e-07
	Treatment	1	0.001101	0.0011010	0.7020	0.4097
	Site*Treatment	10	0.005391	0.0005391	0.3437	0.9597
	Residuals	26	0.040775	0.0015683		
K	Site	10	159676	15967.6	3.5237	0.004733
	Treatment	1	1862	1862.0	0.4109	0.527129
	Site*Treatment	10	25198	2519.8	0.5561	0.833727
	Residuals	26	117820	4531.5		
Mg	Site	10	424354	42435	13.7409	5.264e-08
	Treatment	1	624	624	0.2021	0.6568
	Site*Treatment	10	26665	2667	0.8634	0.5766
	Residuals	26	80294	3088		
Mn	Site	10	36.812	3.6812	1.1202	0.3848
	Treatment	1	0.010	0.0099	0.0030	0.9566
	Site*Treatment	10	44.800	4.4800	1.3633	0.2511
	Residuals	26	85.442	3.2862		
Na	Site	10	17274.0	1727.40	13.9776	4.41e-08
	Treatment	1	117.3	117.29	0.9491	0.33893
	Site*Treatment	10	2845.8	284.58	2.3027	0.04274
	Residuals	26	3213.2	123.58		
P	Site	10	50.669	5.0669	1.3267	0.2682
	Treatment	1	0.525	0.5251	0.1375	0.7138
	Site*Treatment	10	23.508	2.3508	0.6155	0.7867
	Residuals	26	99.295	3.8190		
S	Site	10	2425.03	242.503	4.8200	0.0005963
	Treatment	1	0.11	0.111	0.0022	0.9629385
	Site*Treatment	10	553.05	55.305	1.0992	0.3986210
	Residuals	26	1308.12	50.312		
Si	Site	10	6242.0	624.20	11.3029	3.803e-07
	Treatment	1	0.2	0.16	0.0029	0.9575
	Site*Treatment	10	967.0	96.70	1.7511	0.1218
	Residuals	26	1435.8	55.22		
Zn	Site	10	9.2772	0.92772	3.0373	0.01107
	Treatment	1	0.0669	0.06688	0.2190	0.64373
	Site*Treatment	10	0.6403	0.06403	0.2096	0.99332
	Residuals	26	7.9415	0.30544		

Table 6. Clone genotypes with overall frequency and areas in which the clones are found

Clone Number	Genotype	Frequency	Areas Found
1	00000000001g	9	6, 11, 14,26
2	00000000101g	3	11
3	00000001001g	4	11, 14
4	00000001101g	3	10
5	00000100001g	12	6, 12, 14, 16, 21
6	00000101001g	3	14, 16, 26
7	00000110001g	3	12
8	00010001001g	3	27
9	01000000001g	37	7, 9, 11, 12, 14, 20, CEM
10	01000001001g	15	11, 12, 14, 20, 21, 25
11	01000010001g	3	14
12	01000011001g	2	21
13	01000100001g	6	7, 11, 12, 14, 20, 26
14	01000101001g	12	11, 14
15	01100100001g	2	8, 16
16	01100101001g	2	8
17	10100101001g	2	12
18	11000111001g	2	6
19	00000011001g	1	10
20	00000100101g	1	11
21	00100000001g	1	9
22	00100110001g	1	9
23	01000000101g	1	11
24	01000001100g	1	10
25	01000010111g	1	9
26	01000111001g	1	6
27	01001001001g	1	11
28	01010011001g	1	14
29	01100001101g	1	8
30	01100110001g	1	9
31	01100111001g	1	8

[a]Numbering system from Garvon(2005)); Cem = St. James Cemetery near Bishop, TX.

Table 7. Clonal and genotypic diversity in sampled areas.

Area Number[a]	**Mowing Study Site**[b]	**Number Genotyped**	**Number of Genotypes**	**Effective Number of Genotypes**	**Genotypic Diversity**
6	Z1	8	4	2.91	0.75
7	---	5	2	1.47	0.40
8	T3	5	4	3.57	0.90
9	N3 or Z2	5	5	5.00	1.00
10		5	3	2.27	0.70
11	N2	22	10	5.90	0.87
14	T1	27	10	4.53	0.81
12	---	9	6	4.76	0.89
16	---	9	3	1.59	0.42
20	---	3	3	3.00	1.00
21	---	6	3	3.00	0.80
25	---	4	1	1.00	0.00
26	---	3	3	3.00	1.00
27	---	3	1	1.00	0.00
StJ	N/A	N/A	22	1	1.00

[a] Numbering system from Garvon(2005); StJ = St. James Cemetery

[b] Mowing management impact site, see Table 4 for descriptions.

Table 8. Probability of genotype frequency differences between pairs of areas based on 10,000 permutations performed in GenoDive (Meirmens and Van Tienderen 2004). Numbers in bold represent those pairs that are significantly different after Bonferoni correction for multiple comparisons.

Area[a]	**14**	**11**	**20**	**9**	**25**	**8**	**26**	**27**	**16**	**21**	**10**	**6**	**7**	**12**
14														
11	0.0017													
20	0.4052	0.8356												
9	0.0032	0.0575	1											
25	0.1573	0.2412	0.1436	0.05										
8	0.001	0.0045	0.4688	0.4485	0.0305									
26	0.1249	0.2364	1	0.9822	0.0308	0.4638								
27	0.0047	0.0113	0.1059	0.1739	0.0285	0.0902	0.1055							
16	0.0002	0.0001	0.0458	0.0106	0.0015	0.0089	0.049	0.0047						
21	0.0197	0.0112	0.2277	0.0255	0.2161	0.0076	0.1124	0.0117	0.0349					
10	0.0008	0.0022	0.1811	0.1684	0.022	0.0892	0.1815	0.0345	0.0008	0.0015				
6	0.0003	0.0149	0.0941	0.0447	0.006	0.0218	0.33	0.0204	0.0029	0.0143	0.012			
7	0.0065	0.6479	0.6428	0.2028	0.0172	0.0346	0.0746	0.0156	0.0005	0.0092	0.0265	0.0052		
12	0.0039	0.0214	0.6816	0.186	0.0949	0.0246	0.2936	0.049	0.0034	0.1591	0.0147	0.0092	0.135	
StJ	**0.0001**	**0.0001**	0.0096	0.0007	**0.0003**	**0.0001**	0.0006	0.0007	**0.0001**	**0.0001**	**0.0001**	**0.0001**	0.1802	**0.0001**

[a] Numbering system from Garvon (2005); StJ = St. James Cemetery near Bishop, TX.

Table 9. Mean percent cover (± standard deviation) of Ambrosia, grasses, forbs, and bare ground in five 0.25-m^2 plots for after prescribed burn at St. James Cemetery, Bishop, TX.

Survey Date	**Site**	**%Ambrosia**	**%Grasses**	**%Forbs**	**%Bare Ground**
July 2013 (Pre-burn)	Burned	4.6 (±2.3)	86.4 (±7.4)	6.8 (±5.5)	2.2 (±2.2)
	Not Burned	8.1 (±2.0)	67.2 (±18.8)	7.9 (±5.2)	16.8 (±16.6)
Sept 2013 (Post-burn)	Burned	8.5 (±3.8)	40.7 (±23)	44.6 (±23.2)	6.2 (±2.5)
	Not Burned	17.7 (±11.6)	44.2 (±22.0)	33.7 (±18.5)	4.4 (±2.2)
August 2014 (Post-burn)	Burned	7.2 (±7.7)	73.3 (±9.6)	16.8 (±7.0)	3.1 (±2.4)
	Not Burned	11.1 (±7.0)	72.6 (±11.2)	12.4 (±10.2)	3.9 (±1.0)

Table 10. Mean change (post-burn – pre-burn) in percent cover (± standard deviation) of Ambrosia, grasses, forbs, and bare ground in five 0.25-m^2 plots for after prescribed burn at St. James Cemetery, Bishop, TX.

	Site	**%Ambrosia**	**%Grasses**	**%Forbs**	**%Bare Ground**
Sept 2013 – July 2013	Burned	3.9 (±2.8)	-45.7 (±17.6)	37.8 (±20.6)	4.0 (±2.5)
	Not Burned	9.6 (±10.6)	-23.0 (±16.3)	25.8 (±15.3)	-12.4 (±214.9)
Aug 2014 – July 2013	Burned	2.6 (±7.6)	-13.1 (±10.9)	10.0 (±4.6)	0.9 (±2.6)
	Not Burned	3.0 (±7.2)	3.0 (±24.6)	4.7 (±15.1)	-10.7 (±15.9)
Results of ANOVA	**Treatment**	F = 3.10 P = 0.077	F = 4.73 P = 0.027	F = 0.96 P = 0.405	
	Time	F = 2.565 P = 0.1315	F = 20.34 P = 0.0005	F = 13.98 P = 0.002	
	Interaction	F = 5.570 p = 0.017	F = 1.341 p = 0.293	F = 0.86 P = 0.445	

Table 11. Mean percent cover (± standard deviation) of Ambrosia, grasses, forbs, and bare ground per 0.25-m^2 plot under three mowing regimes in July 2014.

Mowing Regime	**Site**	**%Ambrosia**	**%Grasses**	**%Forbs**	**%Bare Ground**
Not Mowed	N1	10.7 (±7.3)	39.6 (±40.7)	2.2 (±3.9)	47.5 (±39)
	N2	16.6 (±3.2)	72.8 (±5.8)	4.2 (±2.2)	6.4 (±7.3)
	N3	3.1 (±3.4)	81.4 (±24)	6.2 (±7.3)	9.3 (±19.4)
Mean (± s.d.)		**10.1 (±7.3)**	**64.6 (±31.5)**	**4.2 (±3.6)**	**21.1 (±30.5)**
Tractor	T1	10.9 (±1.1)	80.1 (±5.7)	5.4 (±2.6)	3.6 (±2.8)
	T2	16.2 (±7.9)	60.0 (±22)	17.9 (±22.1)	5.9 (±7.2)
	T3	7.6 (±7.2)	9.8 (±3.7)	7.5 (±3.9)	75.1 (±6.8)
Mean (± s.d.)		**11.6 (±6.8)**	**49.9 (±33)**	**10.3 (±13.3)**	**28.2 (±34.8)**
Zero-Turn	Z1	2.4 (±2.2)	77.0 (±25.6)	3.4 (±2.6)	17.2 (±24.8)
	Z2	12.0 (±10.1)	58.5 (±20.8)	3.5 (±2.2)	26 (±24.4)
	Z3	3.0 (±2.9)	33.5 (±32.3)	5.3 (±2.4)	58.2 (±29.8)
Mean (± s.d.)		**5.7 (±7.3)**	**39.2 (±29.6)**	**3.8 (±2.3)**	**36.3 (±29.6)**
Results of Nested ANOVA		F = 0.86 P = 0.468	F = 0.25 P = 0.785	F = 2.20 P = 0.192	

Table 12. Mean proportion of stems flowering (± standard deviation) under three mowing regimes at the NAS-K. The 2014 data are from the five 0.25-m^2 plots established in July 2014; however, 2015 data are from 10 1-m^2 quadrats randomly chosen along the transect used to establish the original semi-permanent plots.

Mowing Regime	**Site**	**Mean Proportion Flowering 2014**	**Mean Proportion Flowering 2015**
Not Mowed	N1	0.54 (±0.29)	No stems
	N2	0.03 (±0.05)	0.20 (±0.29)
Mean (± s.d.)		**0.28 (±0.33)**	
Tractor	T1	0.03 (±0.01)	0.07 (±0.11)
	T2	0 (±0)	0.15 (±0.18)
	T3	Not found	0.02 (±0.03)
Mean (± s.d.)		**0.002 (±0.005)**	0.08 (±0.14)
Zero-Turn	Z1	0 (±0)	No stems
	Z2	0 (±0)	0.14 (±0.22)
	Z3	0 (±0)	1.00 (±0)
Mean (± s.d.)		**0 (±0)**	0.35 (±0.44)

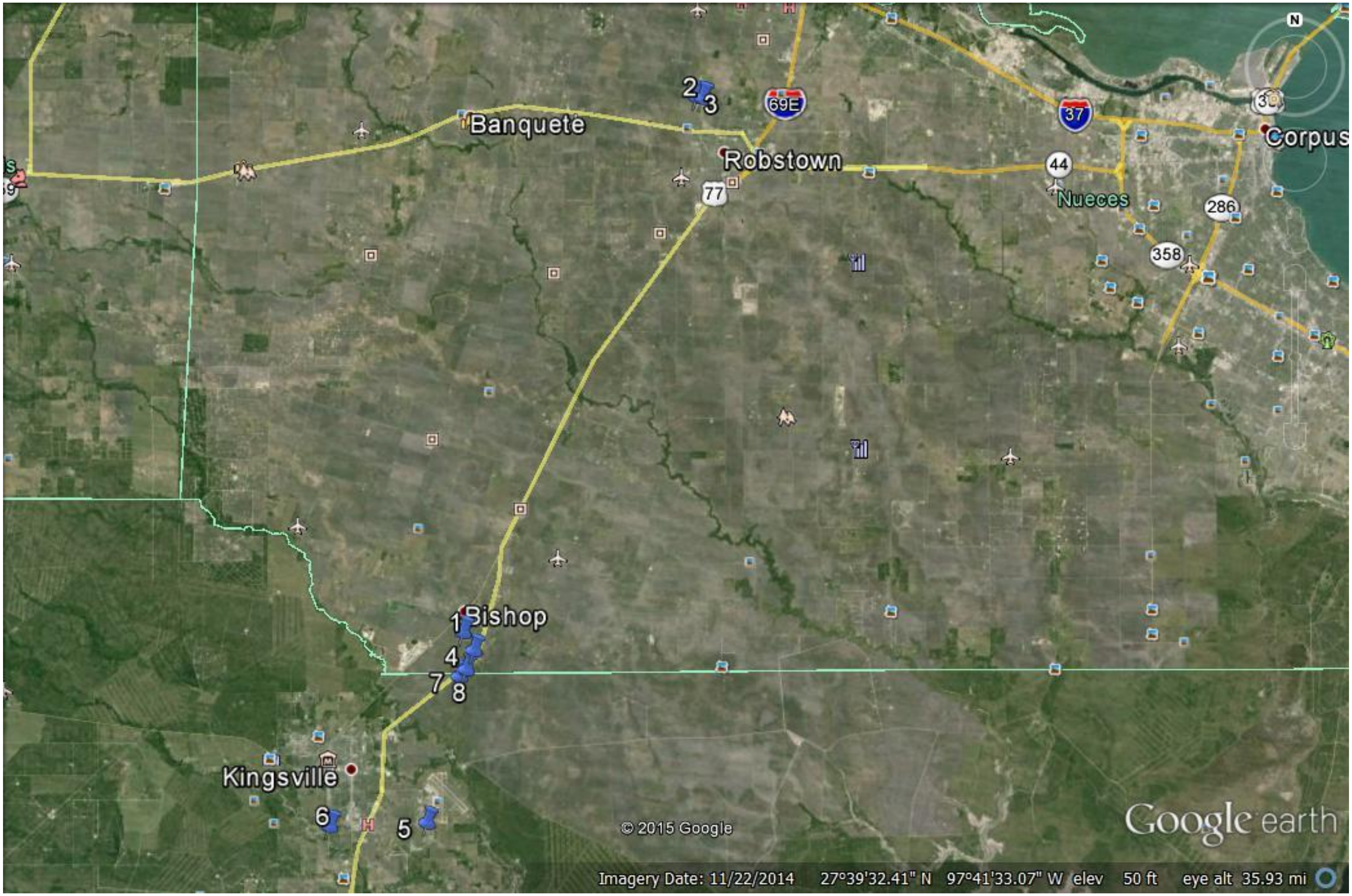


Figure 1. Map of locations visited and surveyed during this study 2012-2015. See Table 1 for location names.

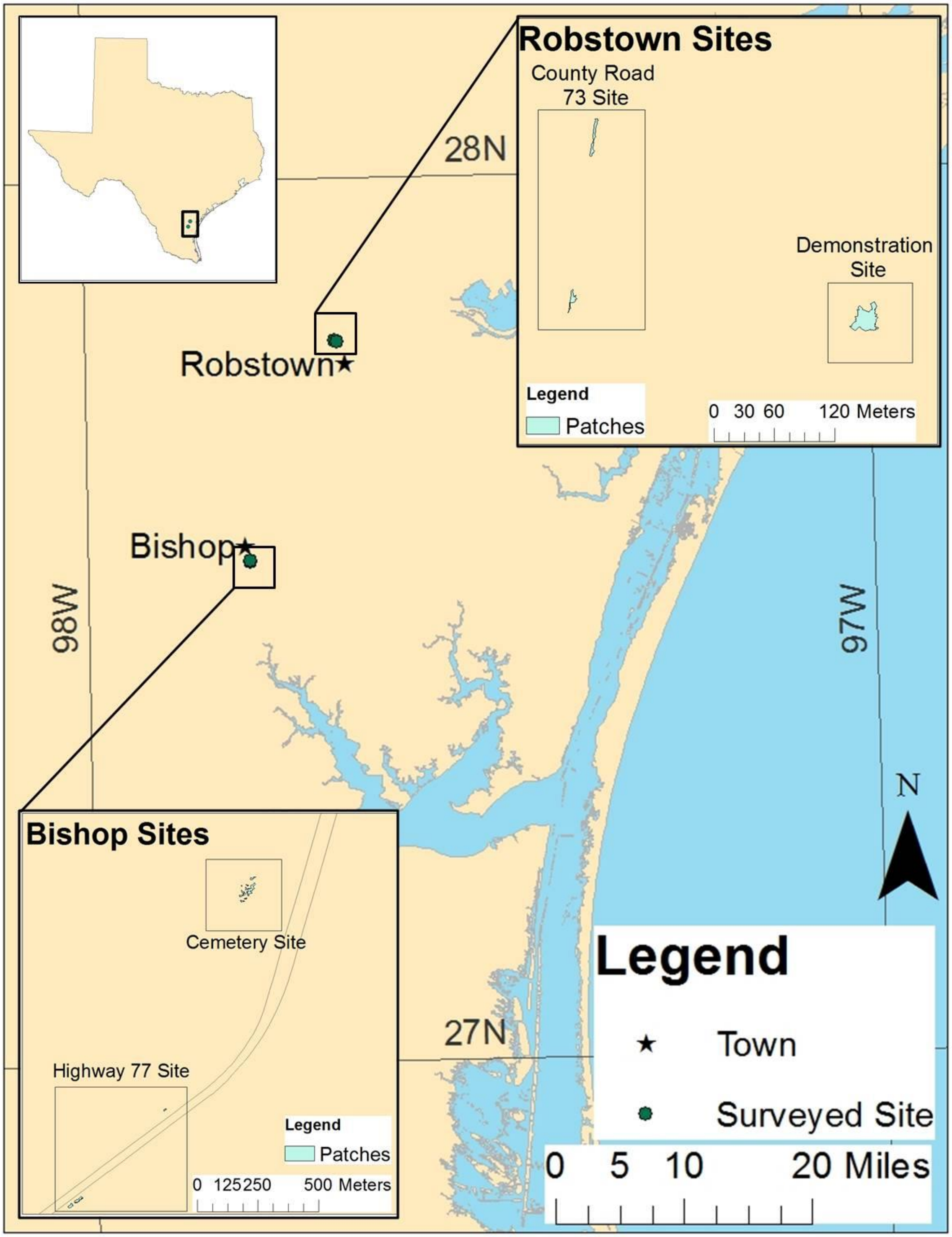


Figure 2. Map of areas surveyed for South Texas Ambrosia on state and private land and extent of its occurrence at each site in June 2013. Percent flowering was also estimated in November 2013 at these sites. (Map made by Alin González.)

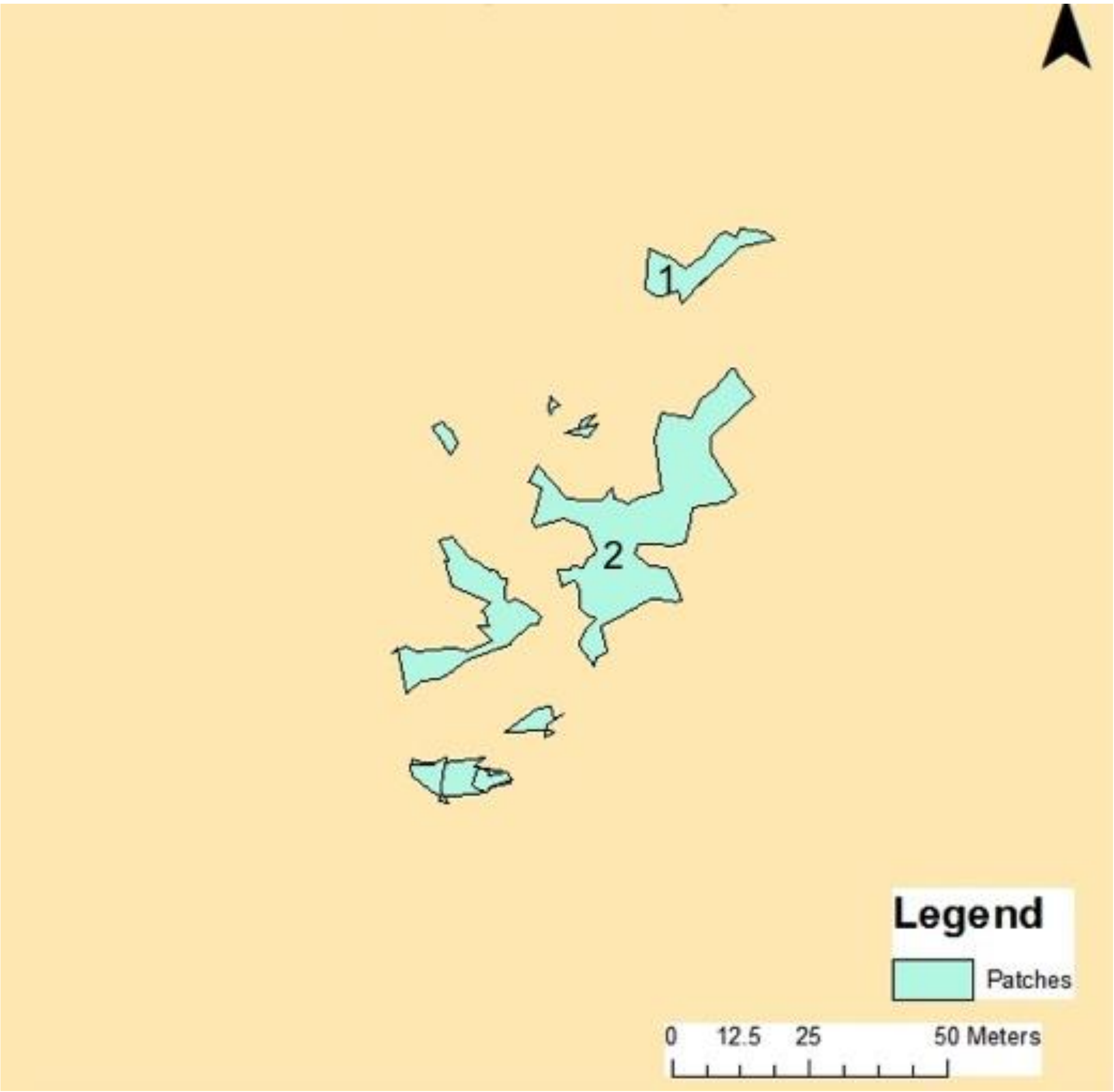


Figure 3. Close-up map of polygons at St. James Cemetery. (See Figure 2 for location within Texas.) The study of the effects of prescribed fire took place at this site. Burned plots were in the polygon 1 and the unburned plots in polygon 2.

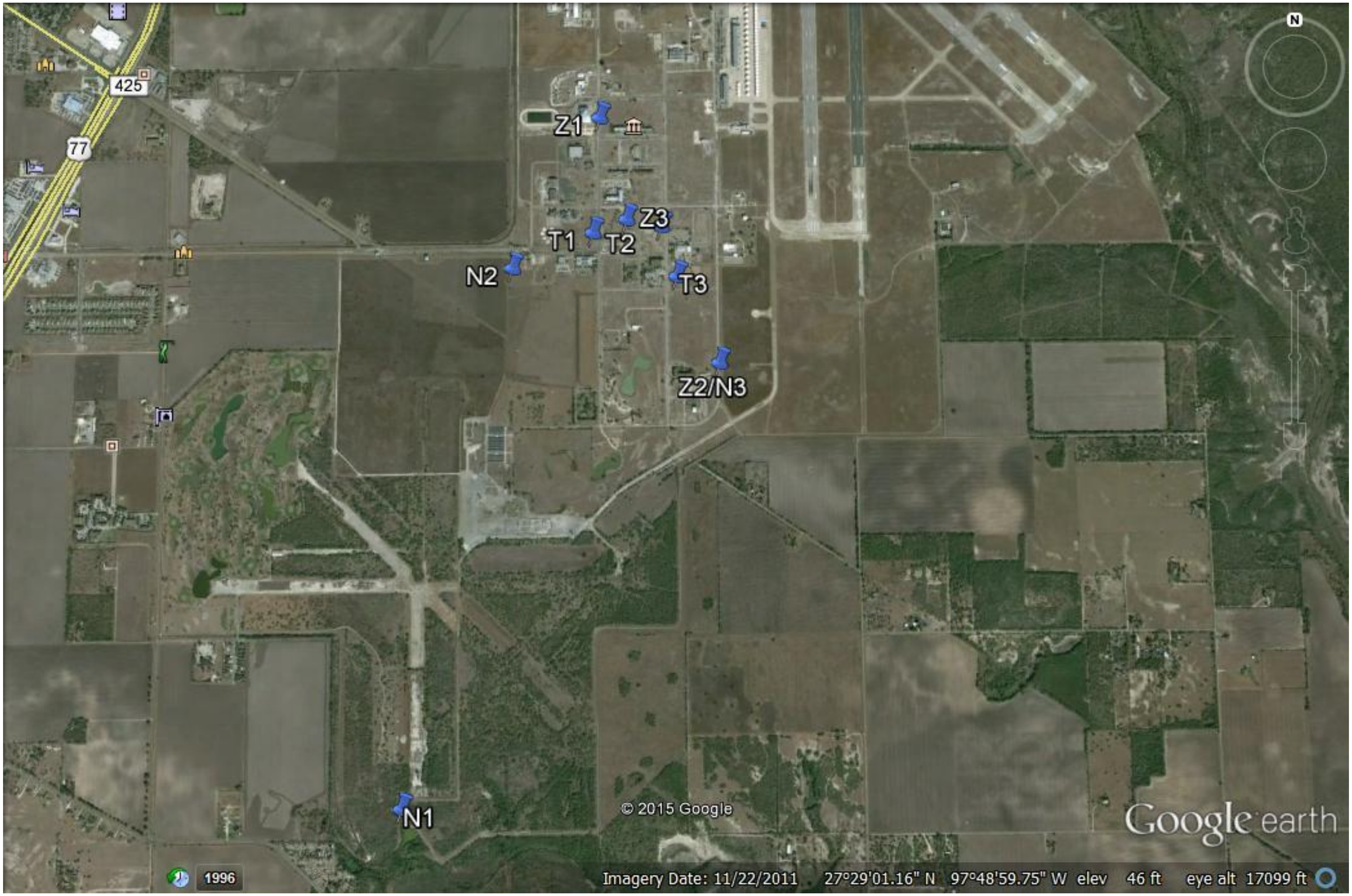


Figure 4. Map of locations on Naval Air Station—Kingsville (NAS-K) involved in the study of the effects of mowing regime. See Table 4 for site codes.

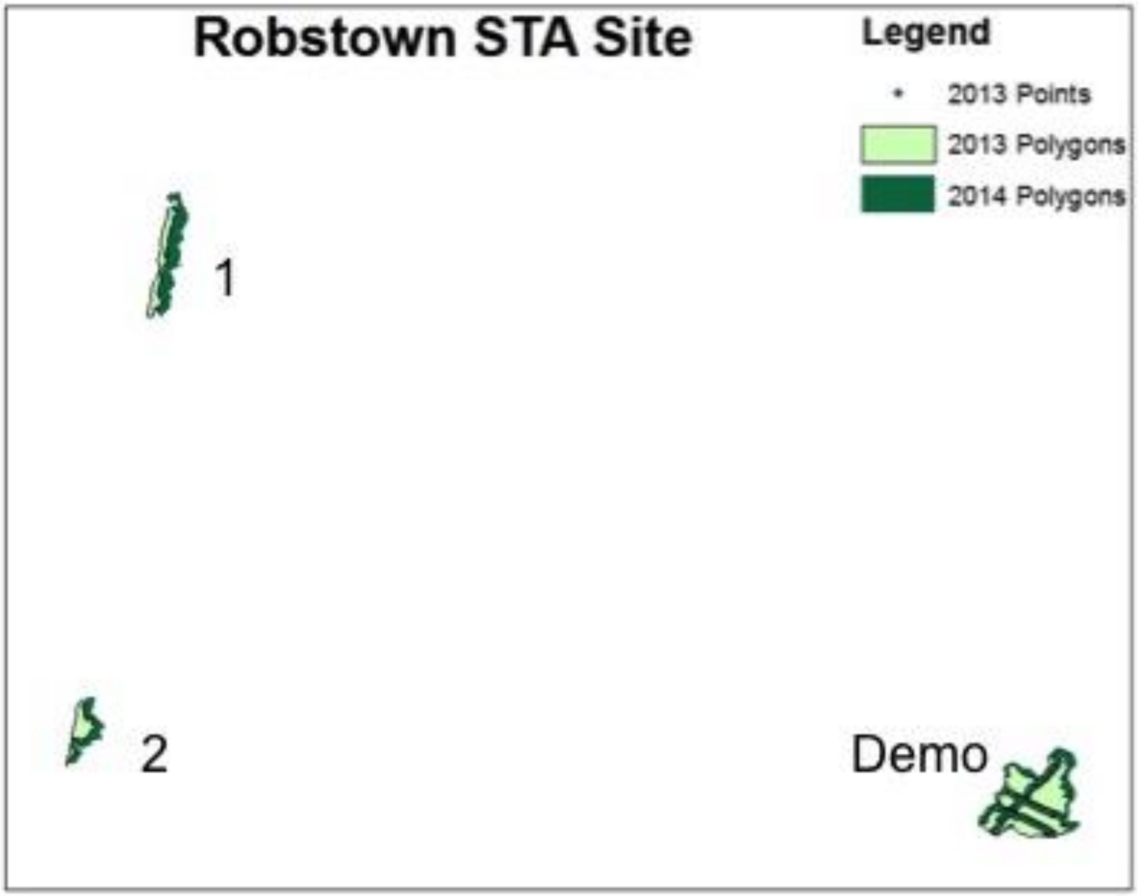


Figure 5. Close-up map of polygons at Robstown County Park. (See Figure 2 for location within Texas.)

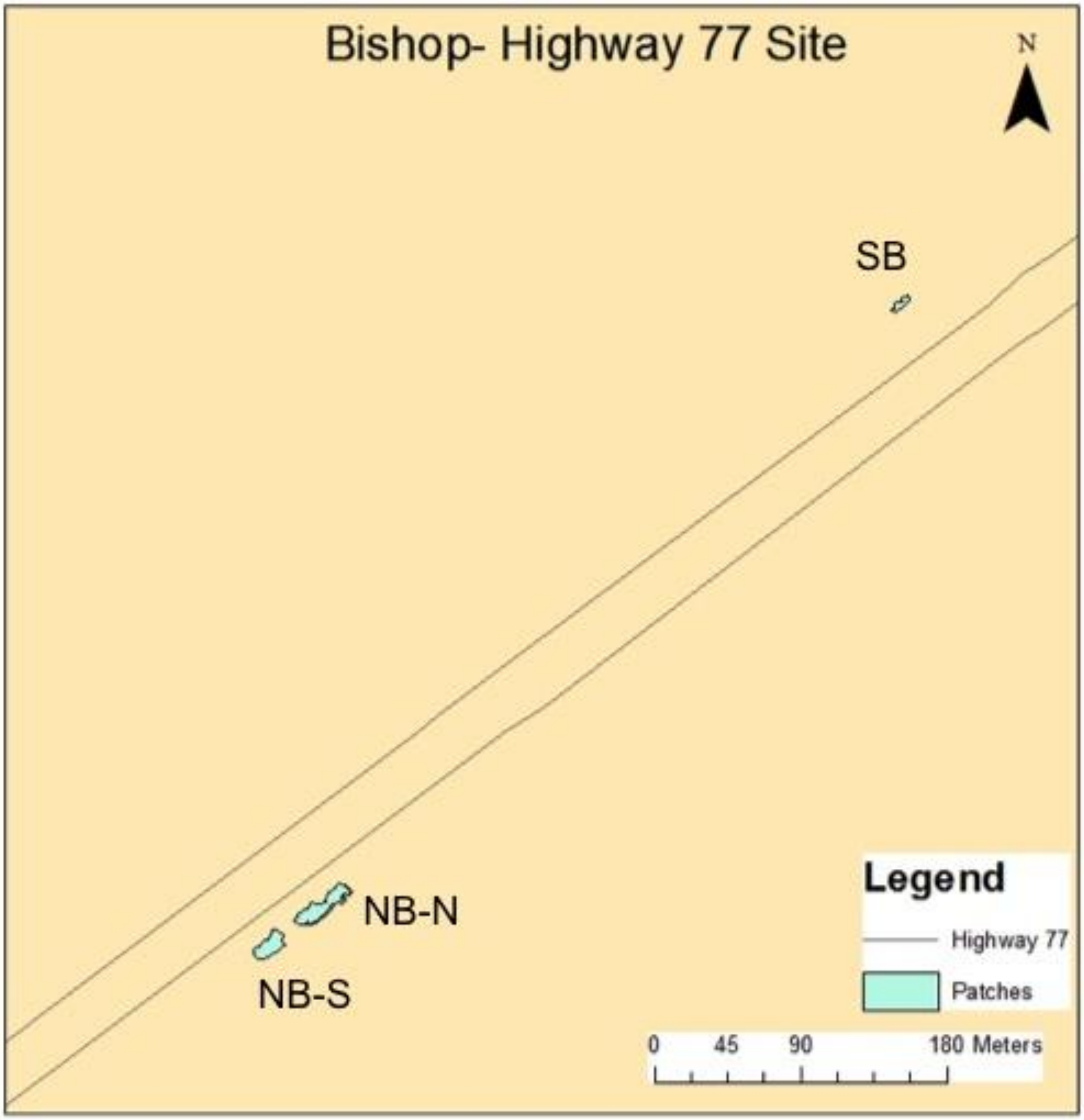


Figure 6. Close-up map of polygons along US 77. (See Figure 2 for location within Texas.)

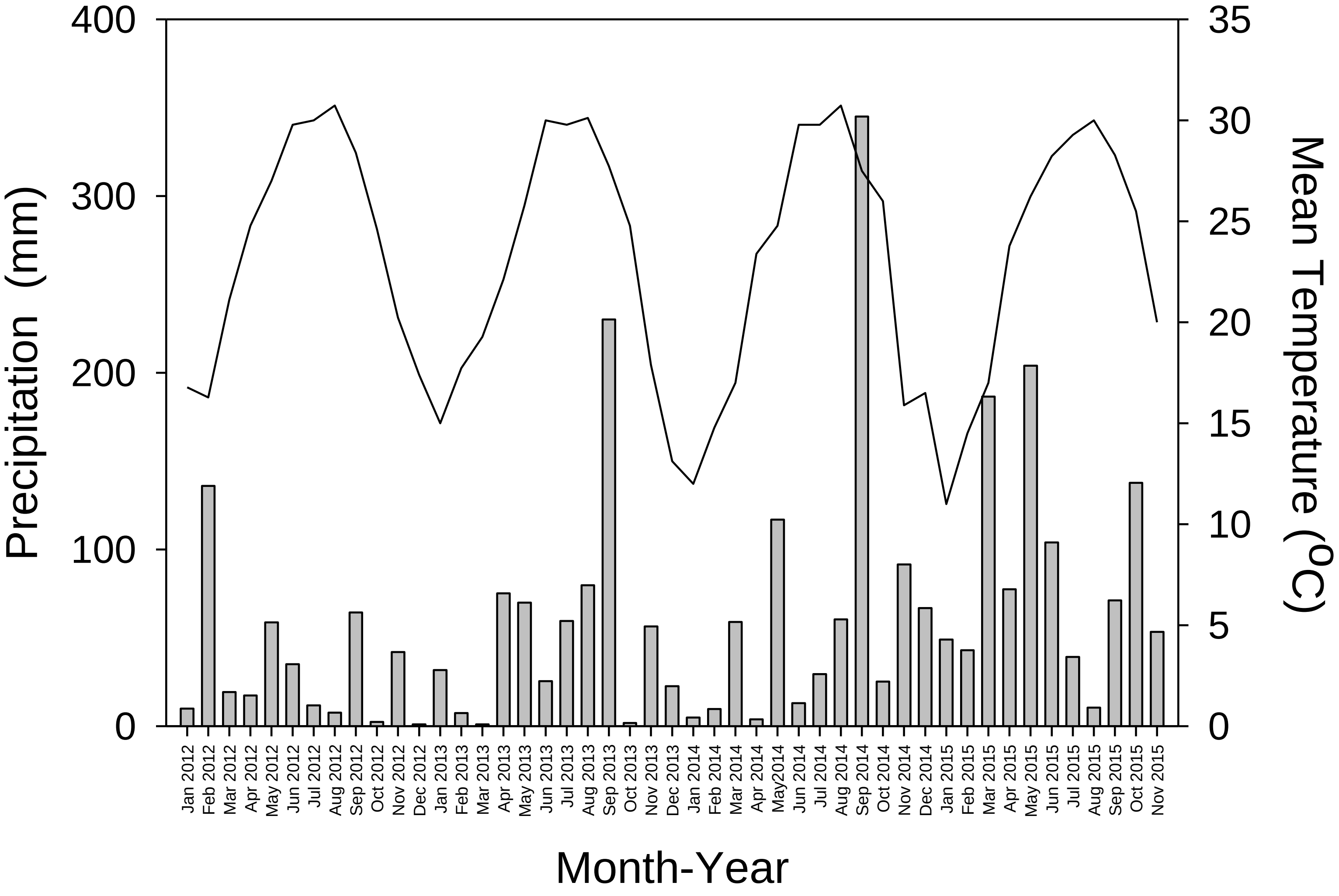


Figure 7. Monthly total precipitation (mm) and mean temperature (^{o}C) from January 2012-November 2015 for Kingsville, TX obtained from the NOAA National Centers for Environmental Information website (http://www.ncdc.noaa.gov/).

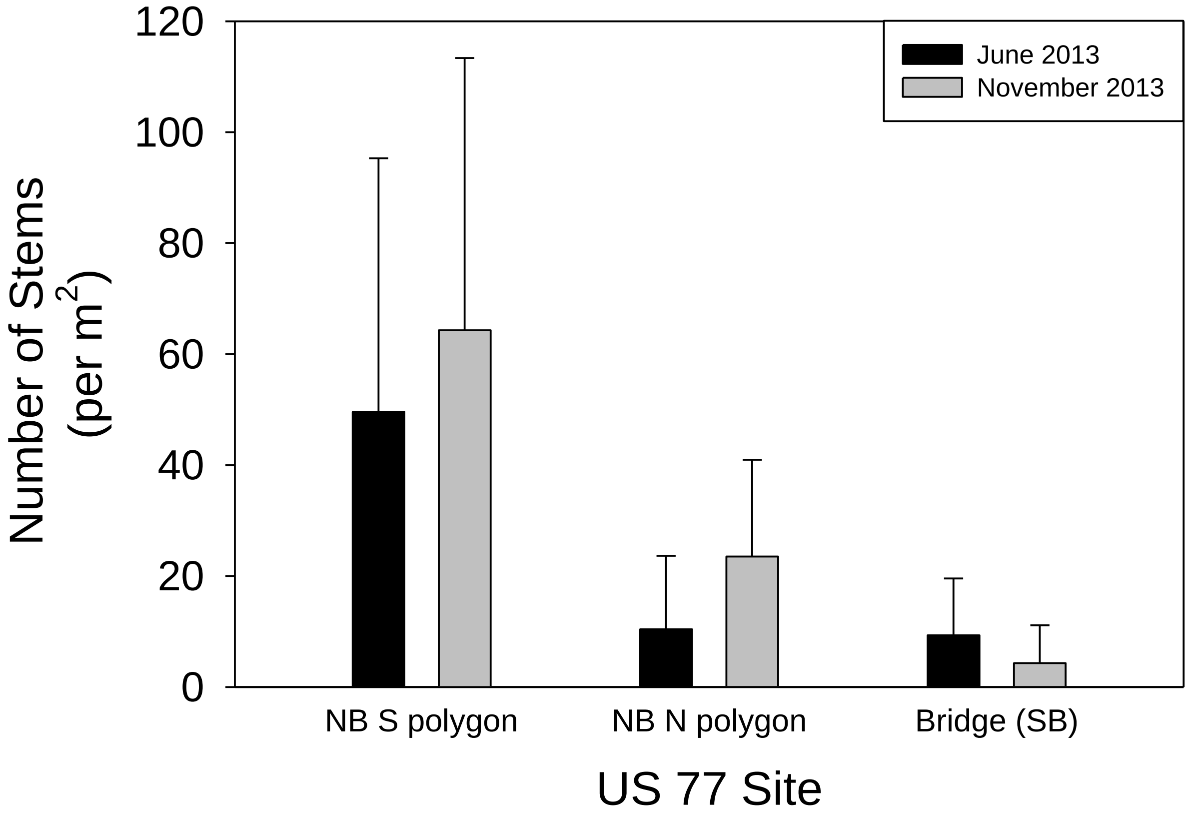


Figure 8. Mean and standard deviation of number of stems per m^2 plot in three polygons for two sites along US 77 near the Kleberg-Nueces County line at two census dates. The NB sites are along the east side of the northbound lane and the SB site is on the west side of the southbound lane of US 77.

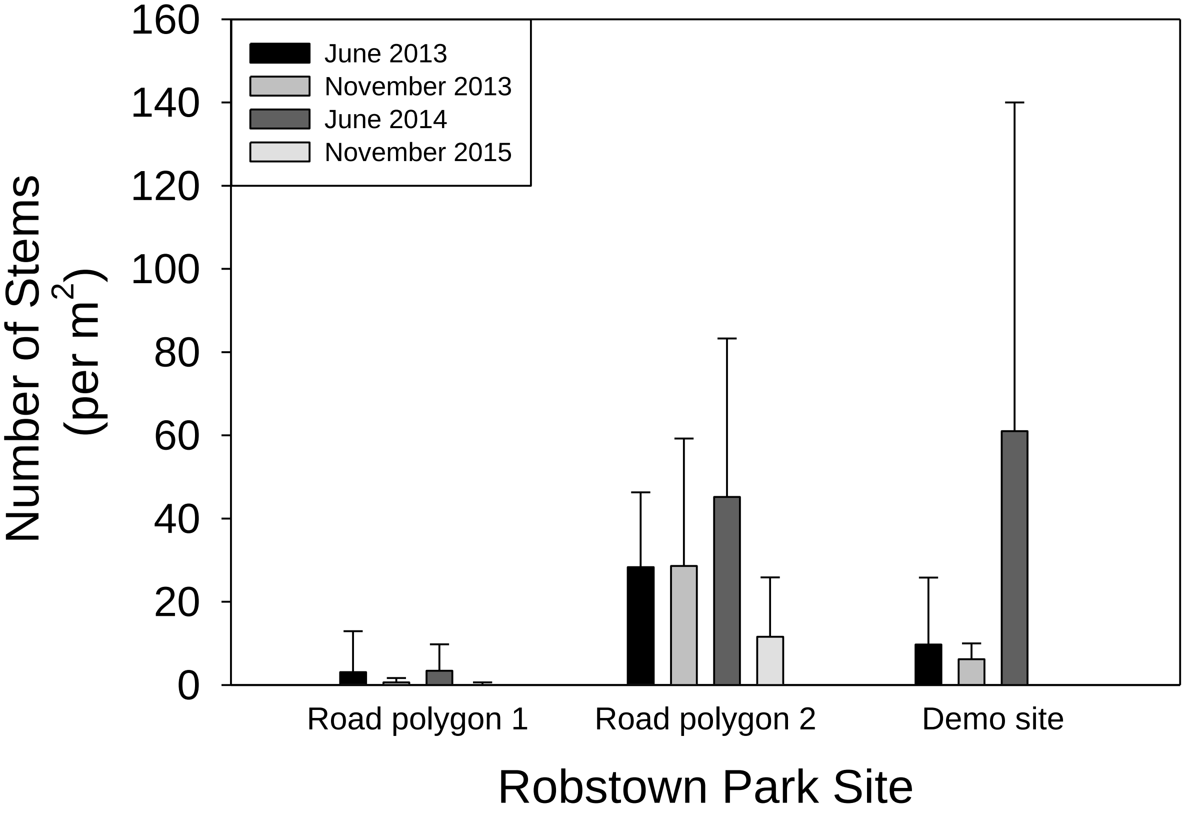


Figure 9. Mean and standard deviation of number of stems per m^2 in two polygons along Co. Rd. 73 and the Demonstration site (Demo) in Robstown Park on four census dates. Note that the Demonstration site was not surveyed in November 2015.

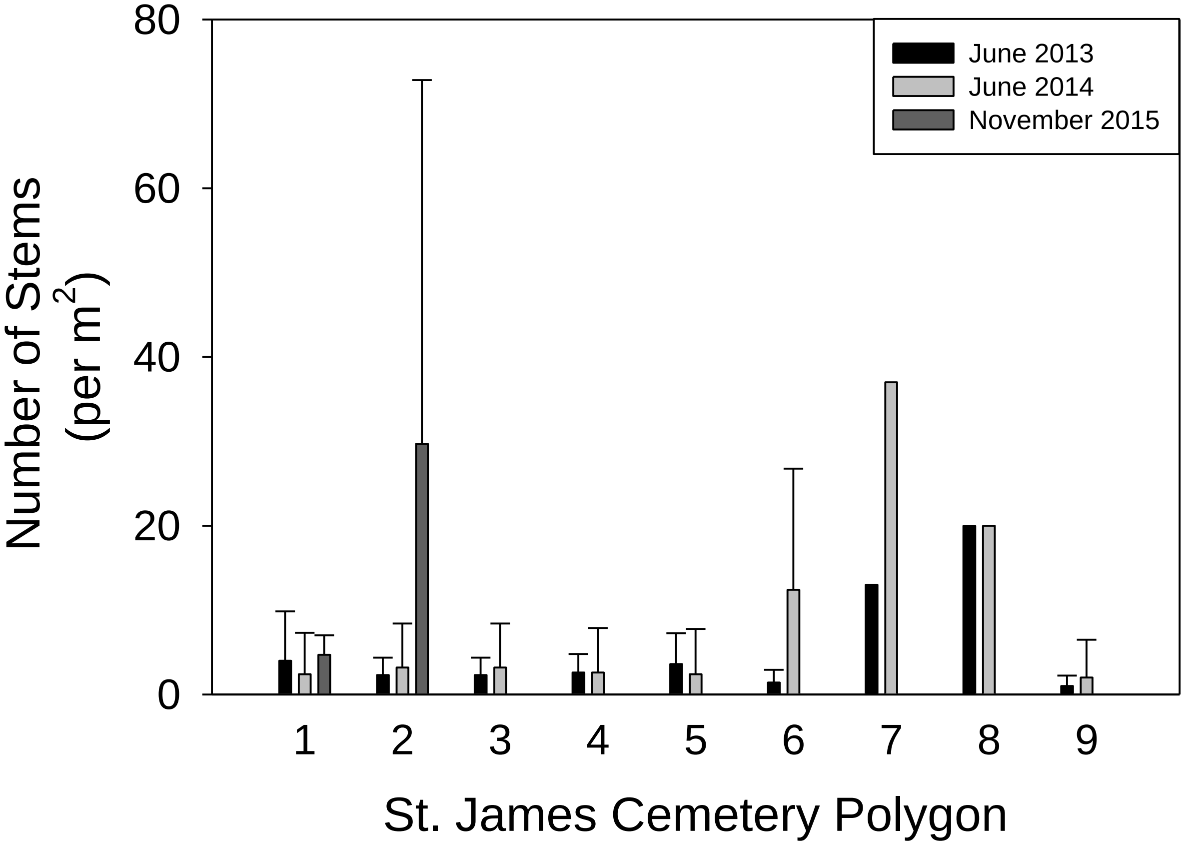


Figure 10. Mean and standard deviation of number of stems per m^2 plots in nine polygons along St. James Cemetery on four census dates. Bars with no standard deviation are total stem count from polygons were less than 1 m^2. For the last census, only polygons 1 and 2 were surveyed. Data for November 2013 survey were only gathered for the prescribed burn study plots and so are not presented here.

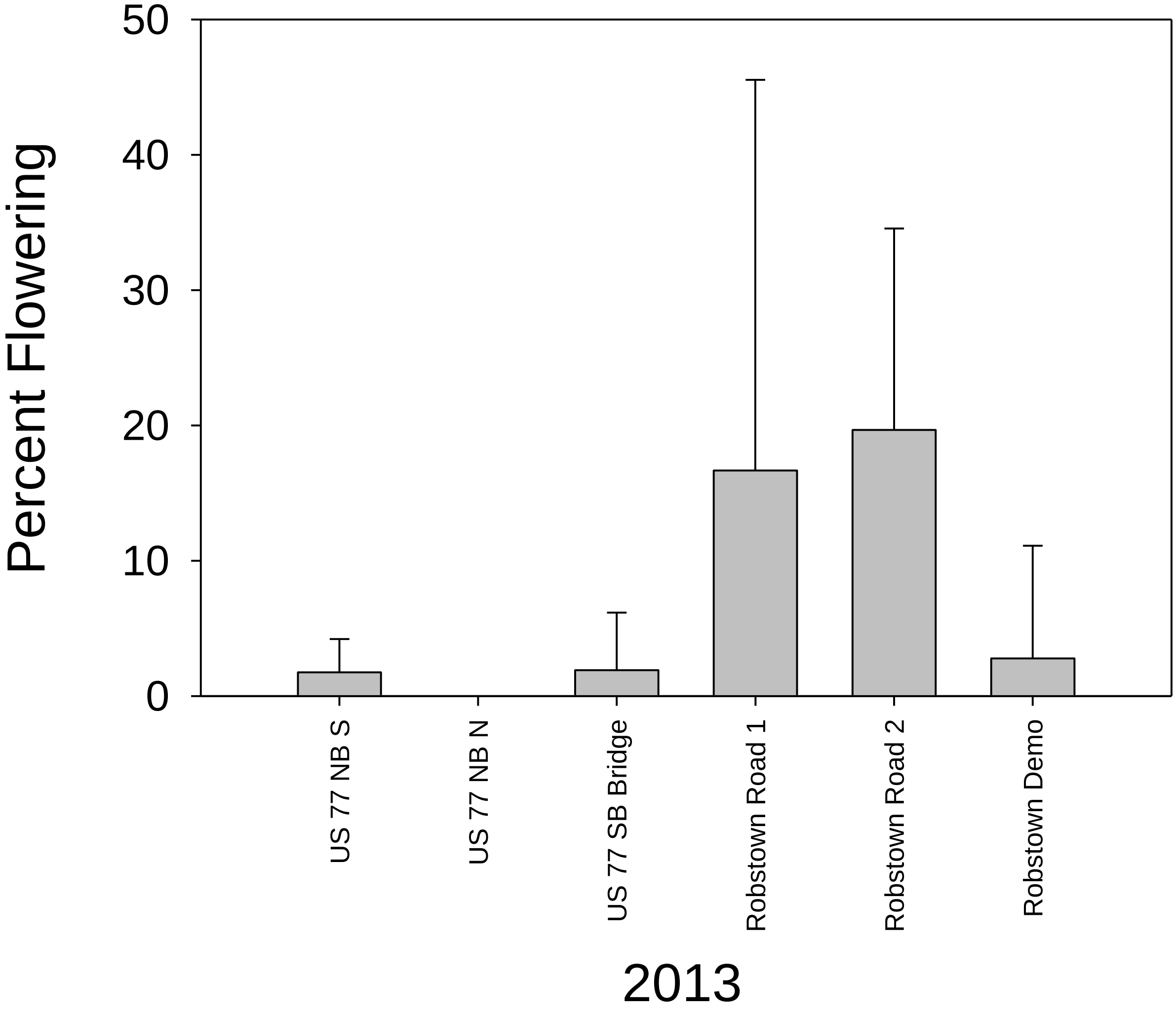


Figure 11. Mean and standard deviation of percent of stems flowering in10 1-m^2 plot in November 2013 in three polygons at the US 77 sites, two Robstown Park polygons along Co. Rd. 73 and the Demonstration Site (Demo) away from the road. Note that the US 77 NB N polygon had no flowering stems because they had been recently mowed; we observed the cut off stem tops with inflorescences at this location. Data do not include quadrats lacking stems.

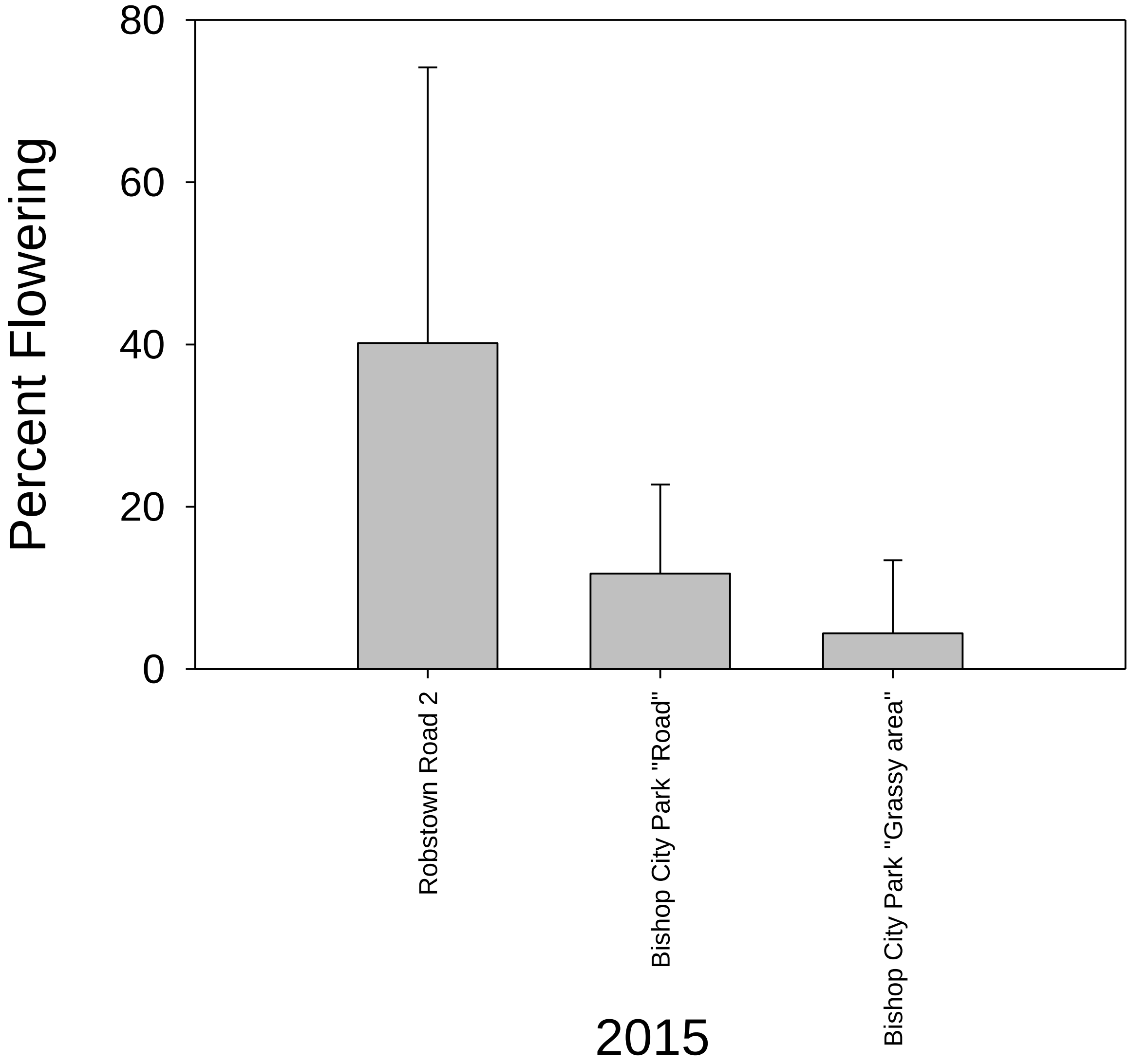


Figure 12. Mean and standard deviation of percent stems flowering in 10 1-m^2 plots in November 2015 in Robstown Park polygon 2 along Co. Rd. 73 and two adjacent areas in Bishop City Park. Note: Robstown Park polygon 1 is not included because no flowering stems were sampled in 10 randomly chosen quadrats, although there were some stems in this polygon.

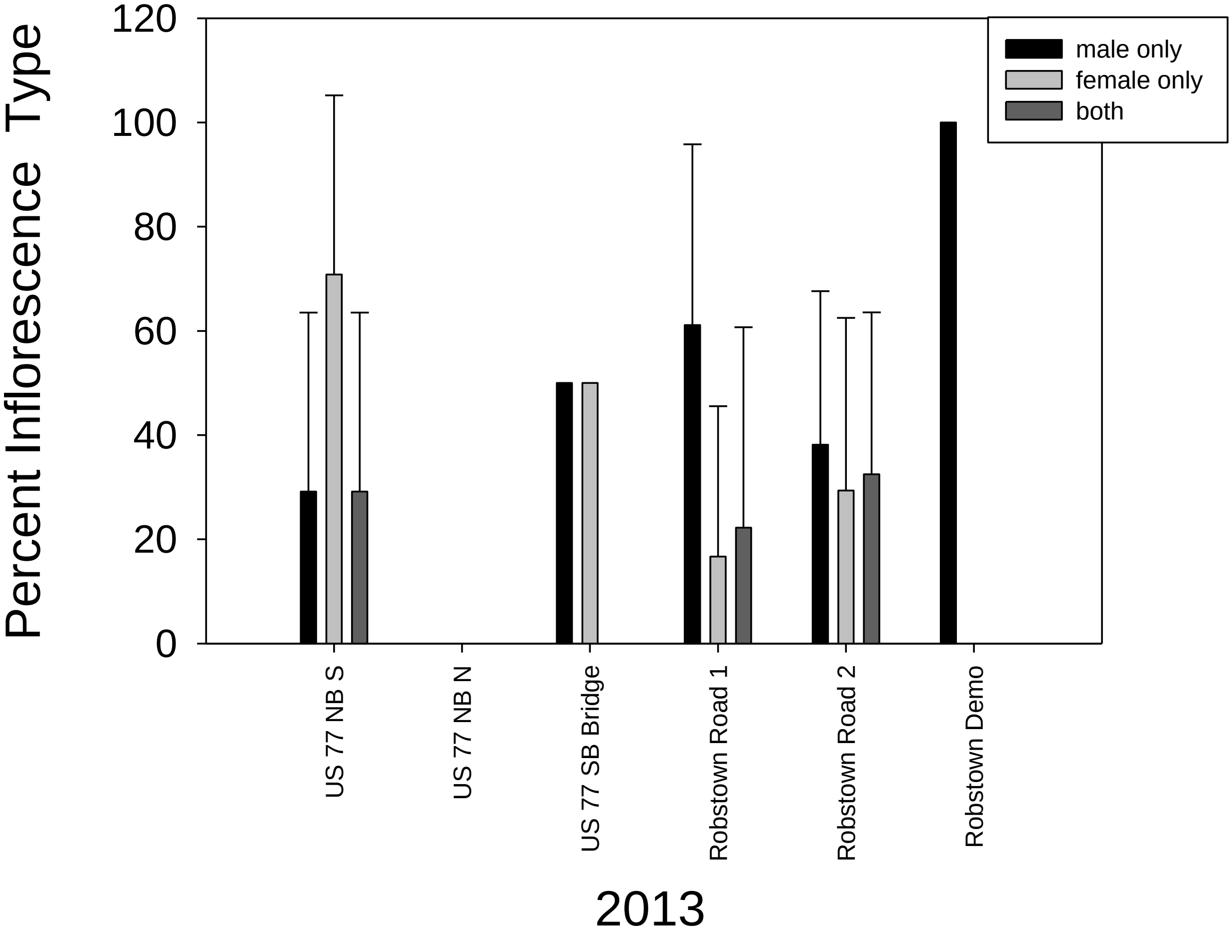


Figure 13. Mean and standard deviation of percent inflorescence type (male only, female only, or both) in 1-m^2 plot in November 2013 in three polygons at the US 77 sites, two Robstown Park polygons along Co. Rd. 73 and the Demonstration Site (Demo) away from the road. Note that the US 77 NB N polygon had no flowering stems because they had been recently mowed; we observed the cut off stem tops with inflorescences at this location. Data do not include quadrats lacking flowering stems, explaining why some bars have no standard deviation.

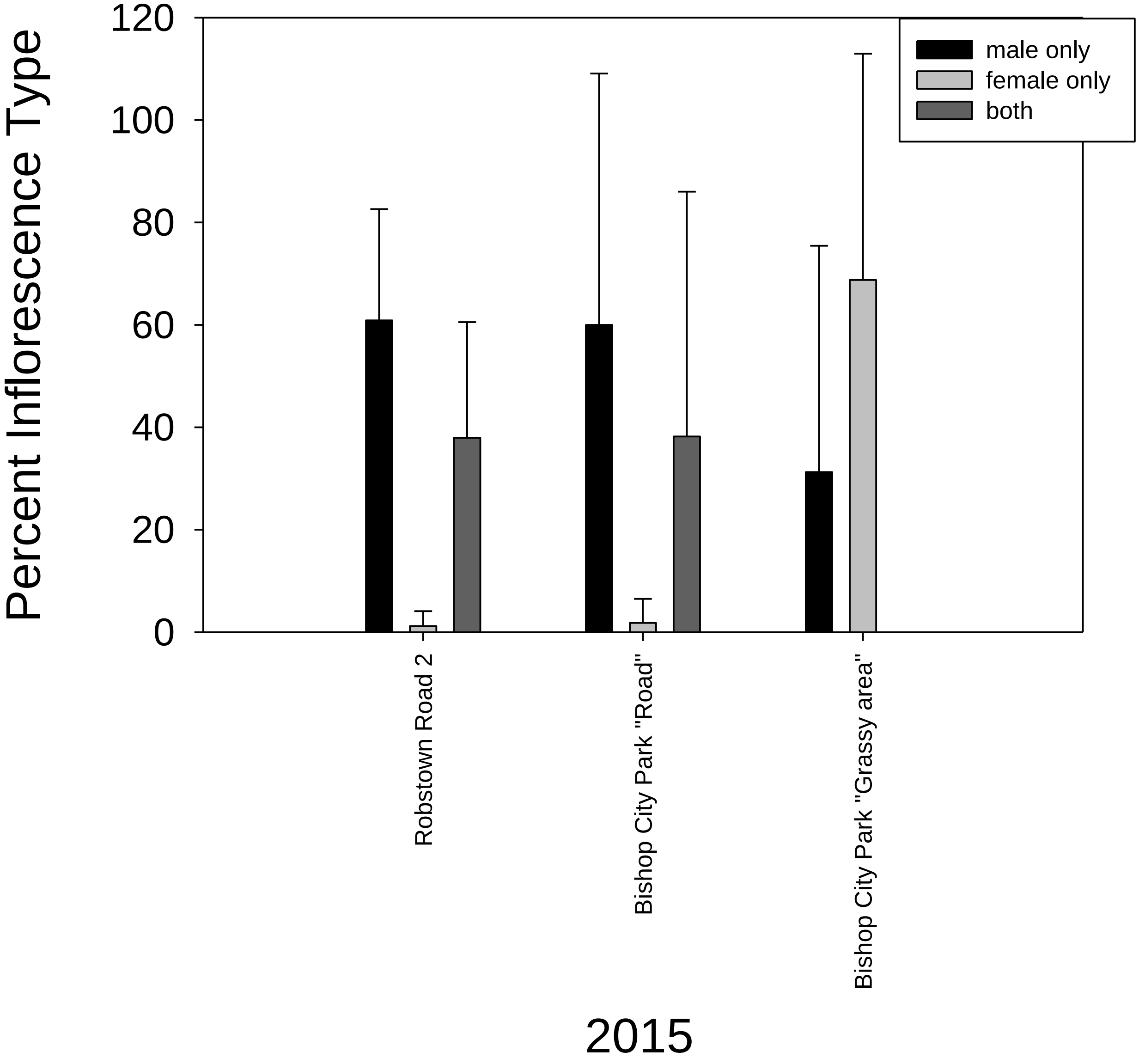


Figure 14. Mean and standard deviation of percent inflorescence type in 10 1-m^2 plots in November 2015 in Robstown Park polygon 2 along Co. Rd. 73 and two adjacent areas in Bishop City Park. Note: Robstown Park polygon 1 is not included because no flowering stems were sampled in 10 randomly chosen quadrats, although there were some stems in this polygon.

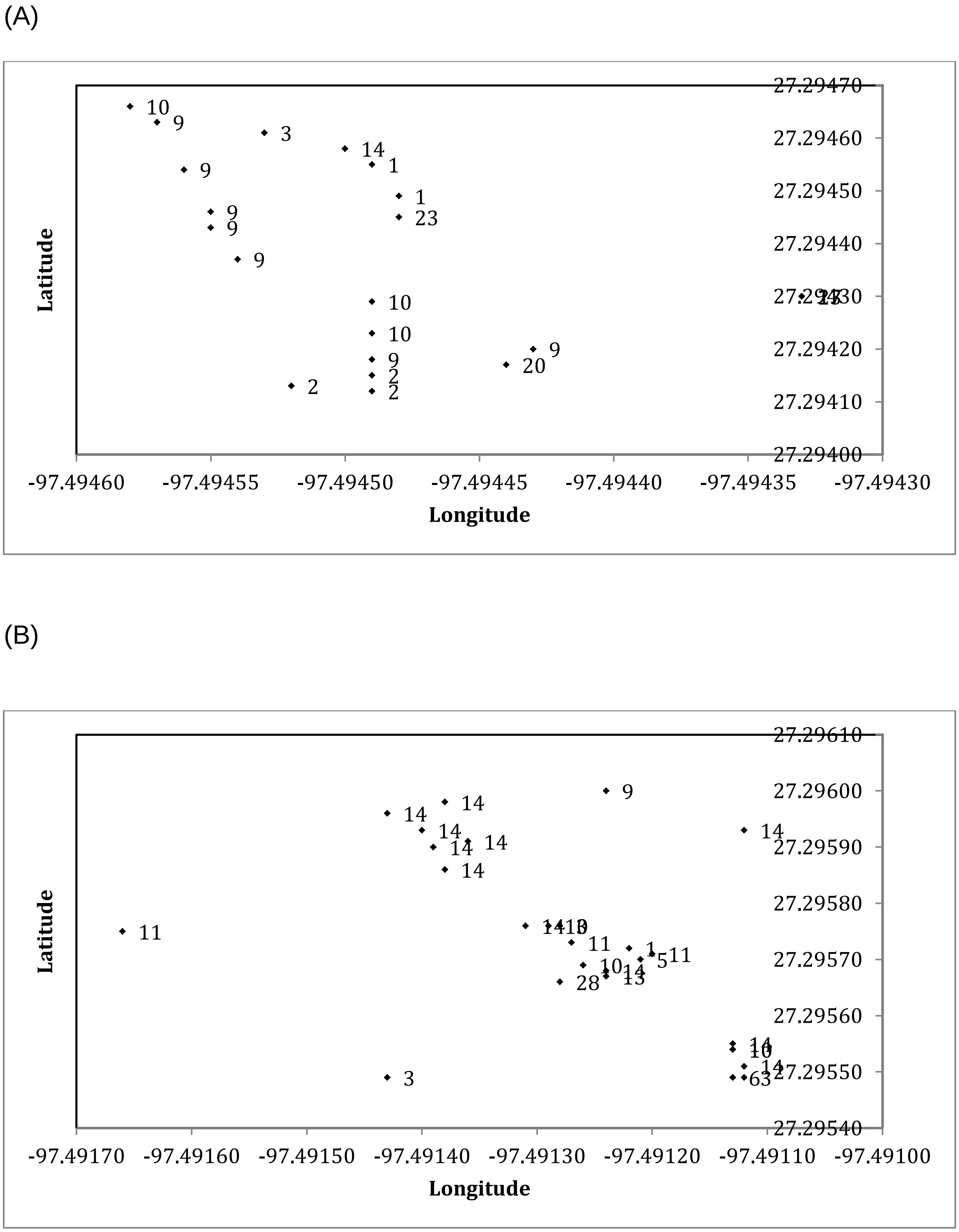


Figure 15. Coordinates of clones for areas 11 (A) and 14 (B). Numbers are clone number (see Table 8).

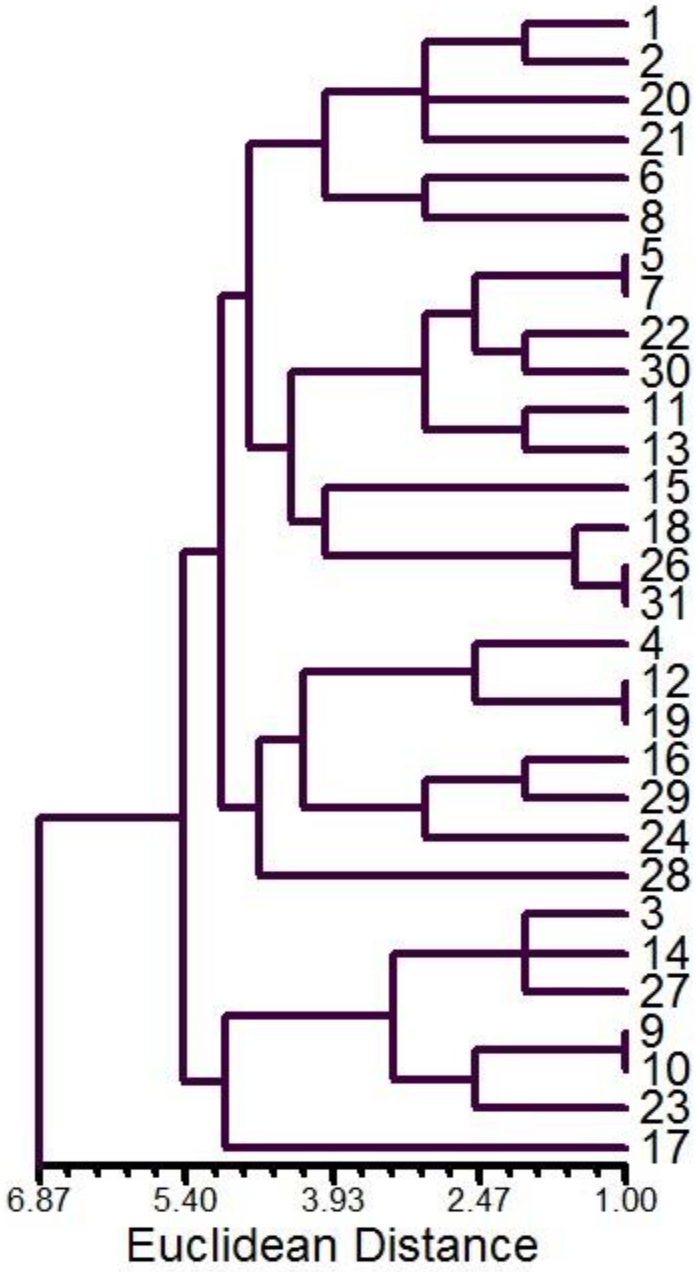


Figure 16. UPGMA phenogram of Euclidean genetic distance between clones based on the number of fragments differences (NTSYS 2.2; Rohfl 1997). Distances were calculated in *GenAlEx 6.5*, which uses the method of Huff et al. (1993).

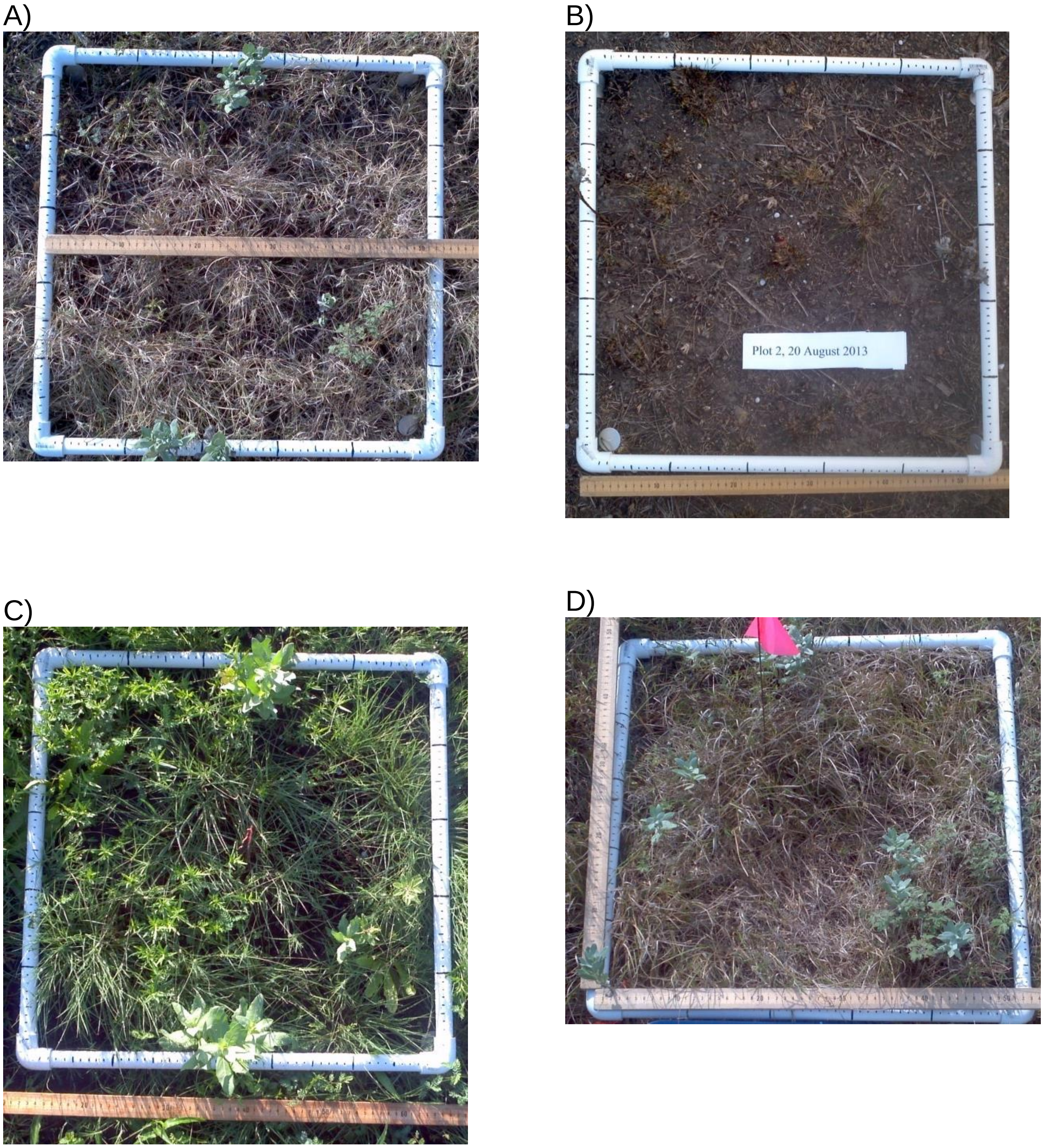


Figure 17. Example of the effects of prescribed burning at St. James Cemetery. Plot 2 in A) July 2013 pre-burn, B) August 2013 post-burn, C) September 2013 post-burn, and D) August 2014 post-burn. Photos taken by Dave Grisé.

Figure 18. Photo of Plot 1 showing reemergence of Ambrosia stems at the base of stems killed by fire. Photo by Dave Grisé taken on August 27, 2013, three weeks after the prescribed burn at St James Cemetery.

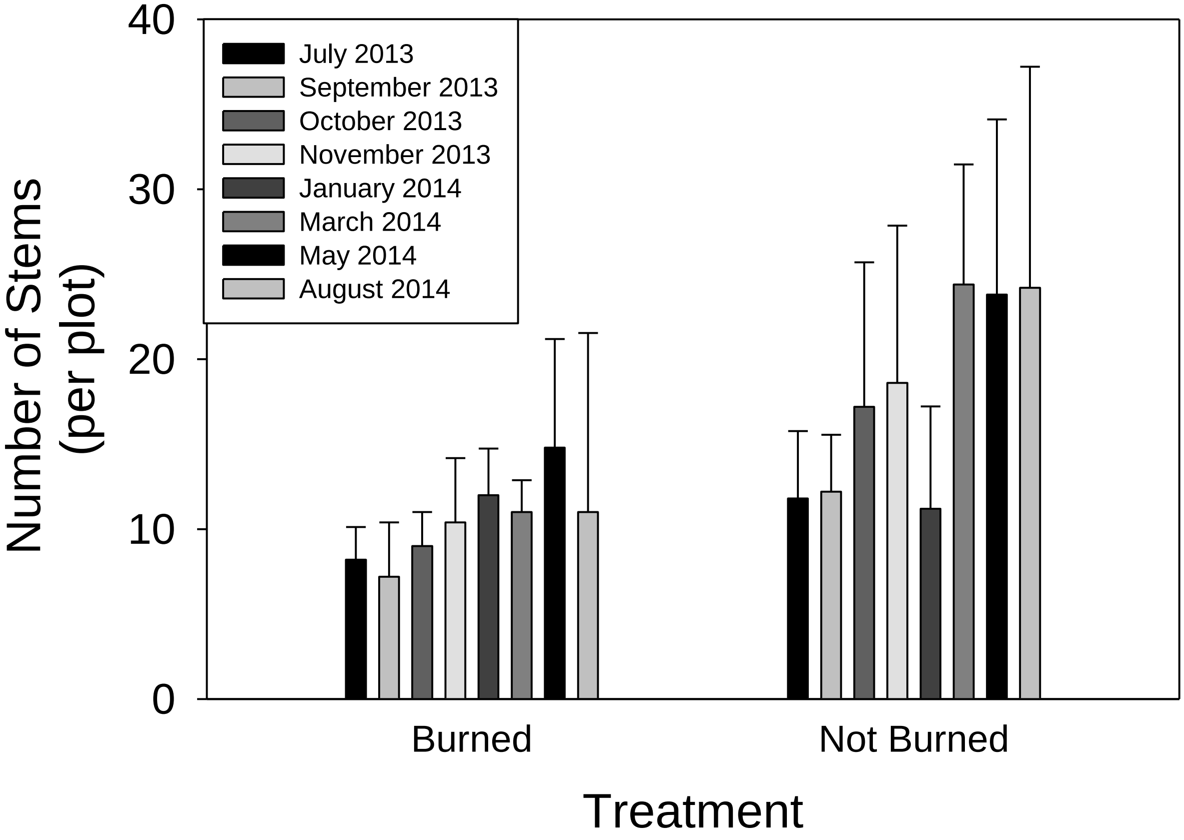


Figure 19. Mean and standard deviation of number of stems in five 0.25-m^2 plots in before (July 2013) and after (all other dates) the prescribed burn at St. James Cemetery on eight census dates.

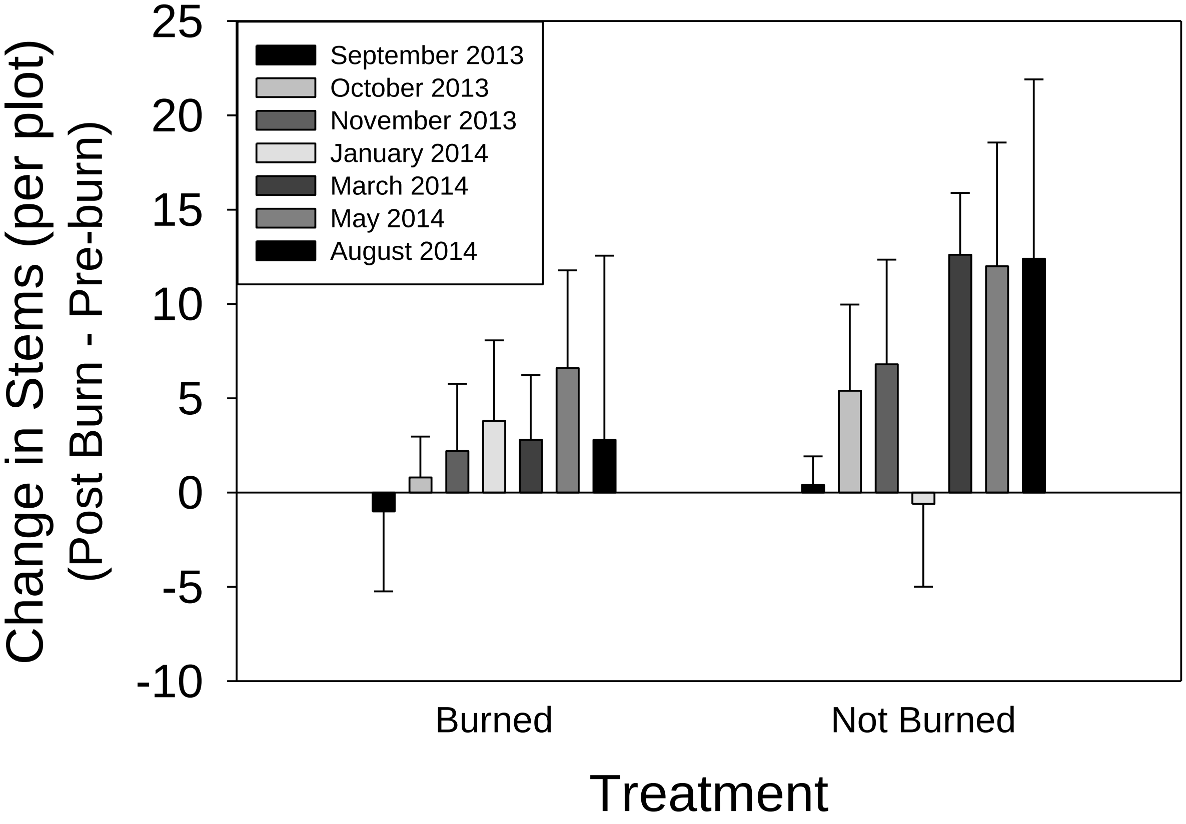


Figure 20. Mean and standard deviation of change in number of stems in five 0.25-m^2 plots between pre-burn census and post burn after the prescribed burn at St. James Cemetery for seven census dates. $F_{treatment}$ = 10.8, $p_{treatment}$ = 0.002; F_{time} = 17.4, p_{time} = 9.1 X 10^{-5}, $F_{interaction}$ = 2.8, $p_{interaction}$ = 0.1.

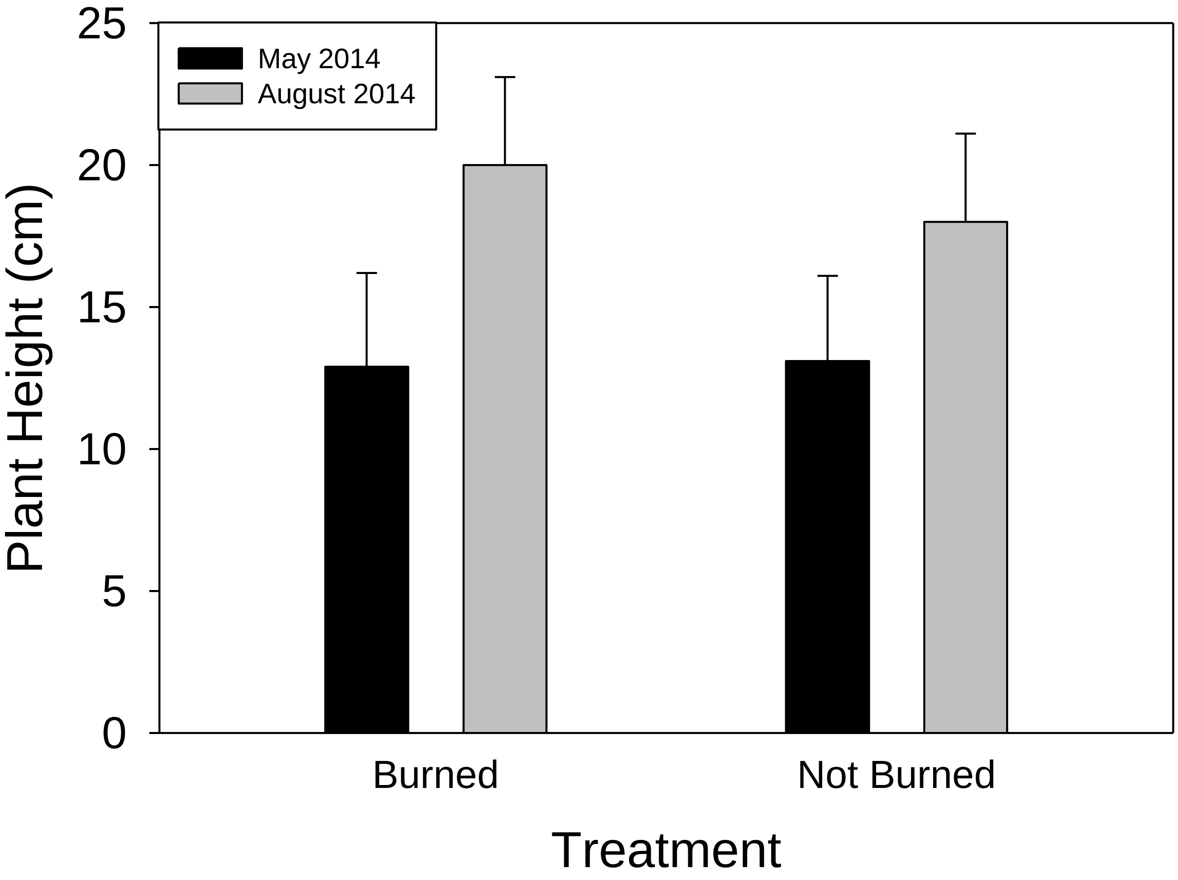


Figure 21. Mean and standard deviation of plant height (cm) of all stems in five 0.25-m^2 plots after the prescribed burn at St. James Cemetery on two census dates. $F_{treatment} = 1.23$, $P_{treatment} = 0.265$; $F_{time} = 311.8$, $P_{time} < 1 \times 10^{-16}$, $F_{interaction} = 10.7$, $P_{interaction} = 0.001$.

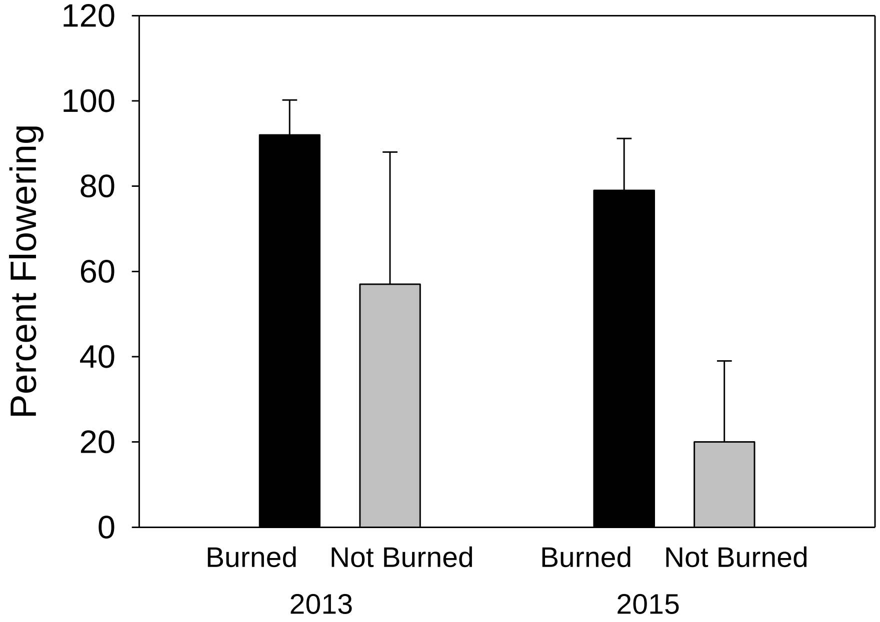


Figure 22. Mean and standard deviation of percent flowering stems of five 0.25-m^2 plots in November 2013 and ten 1-m^2 plots in November 2015 after the August 2013 prescribed burn at St. James Cemetery. 2013: F = 4.6, P = 0.06; 2015: F = 8.4, P = 0.01.

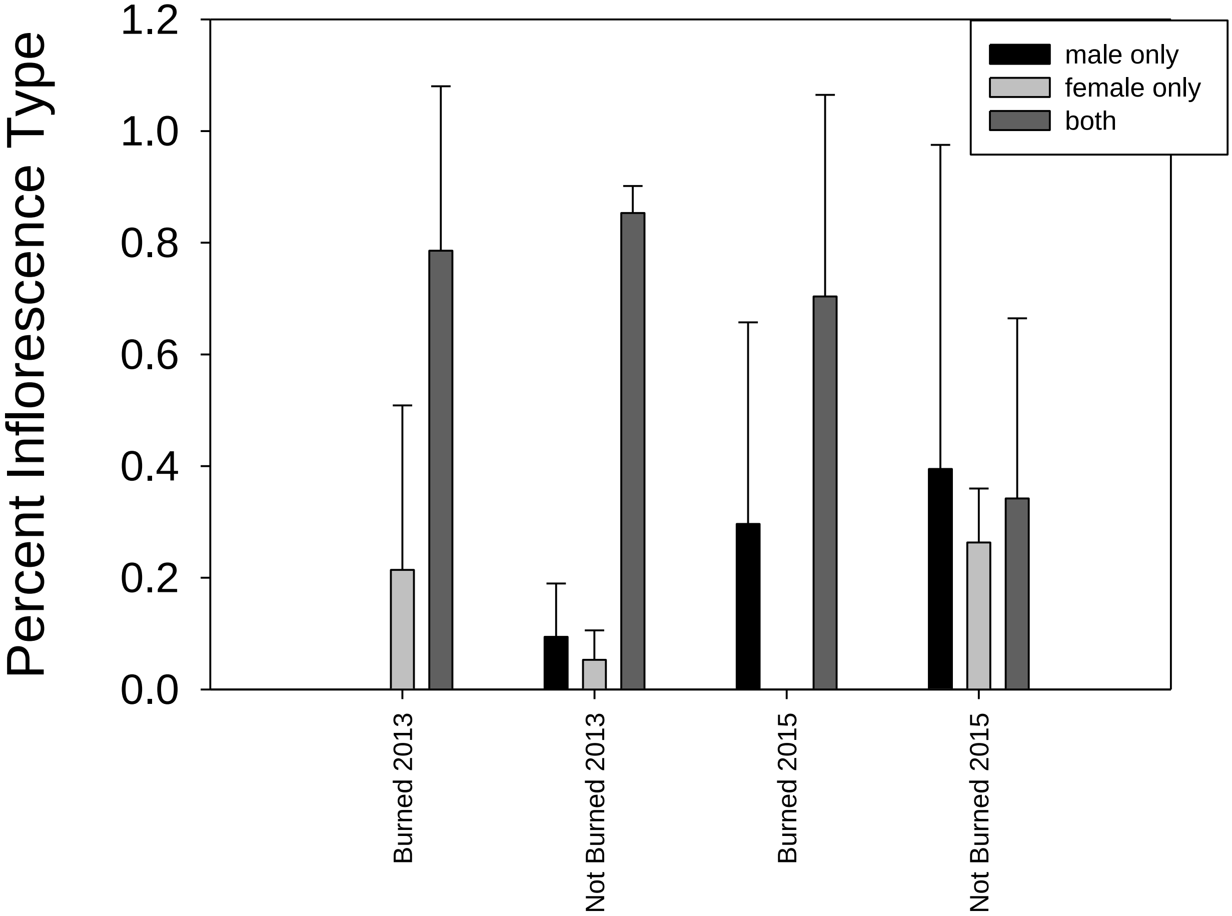


Figure 23. Mean and standard deviation of percent inflorescence type of five 0.25-m^2 plots in November 2013 and ten 1-m^2 plots in November 2015 after the August 2013 prescribed burn at St. James Cemetery.

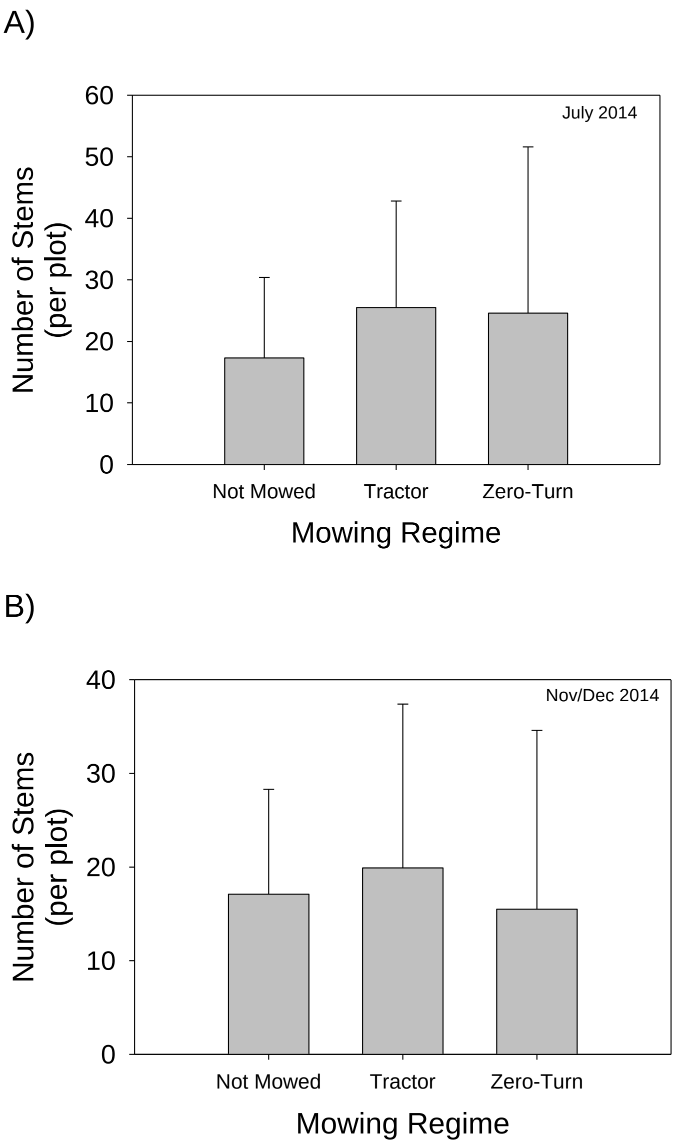


Figure 24. Mean and standard deviation of the number of stems in five 0.25-m^2 plots under three mowing regimes at NAS-K for A) July 2014 and B) Nov/Dec 2014 surveys. July 2014: F = 0.186, P = 0.84; Nov/Dec 2014: F = 0.288, P = 0.764.

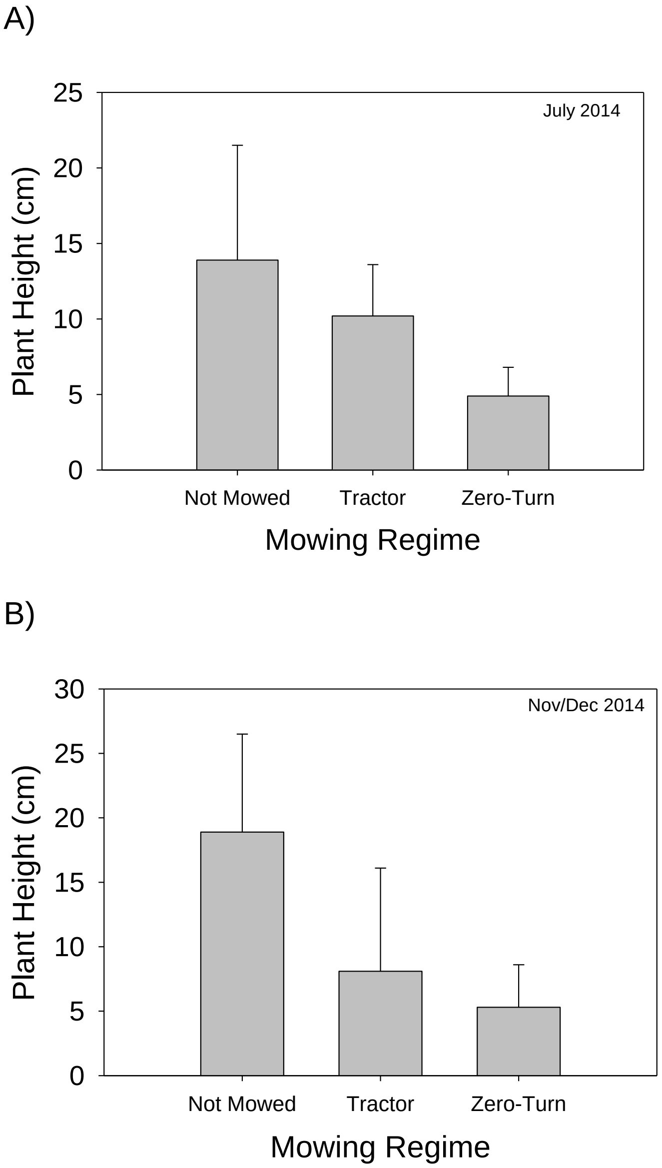
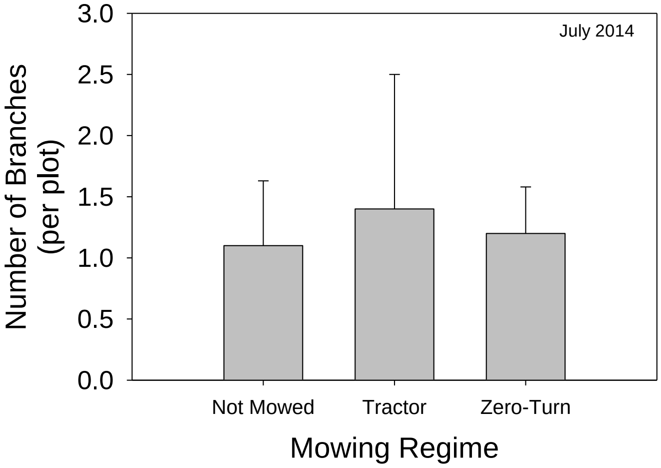


Figure 25. Mean and standard deviation of plant height for all stems in five 0.25-m^2 plots under three mowing regimes at NAS-K for A) July 2014 and B) Nov/Dec 2014 surveys. July 2014: F = 3.04, P = 0.123; Nov/Dec 2014: F = 6.11, P = 0.061.

A)



B)

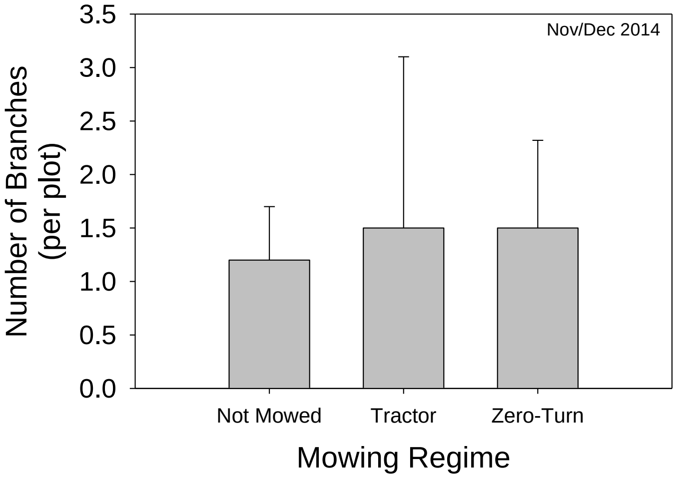


Figure 26. Mean and standard deviation of number of branches per stem for all stems in five 0.25-m^2 plots under three mowing regimes at NAS-K for A) July 2014 and B) Nov/Dec 2014 surveys. July 2014: F = 3.74, P = 0.0.088; Nov/Dec 2014: F = 2.749, P = 0.177.

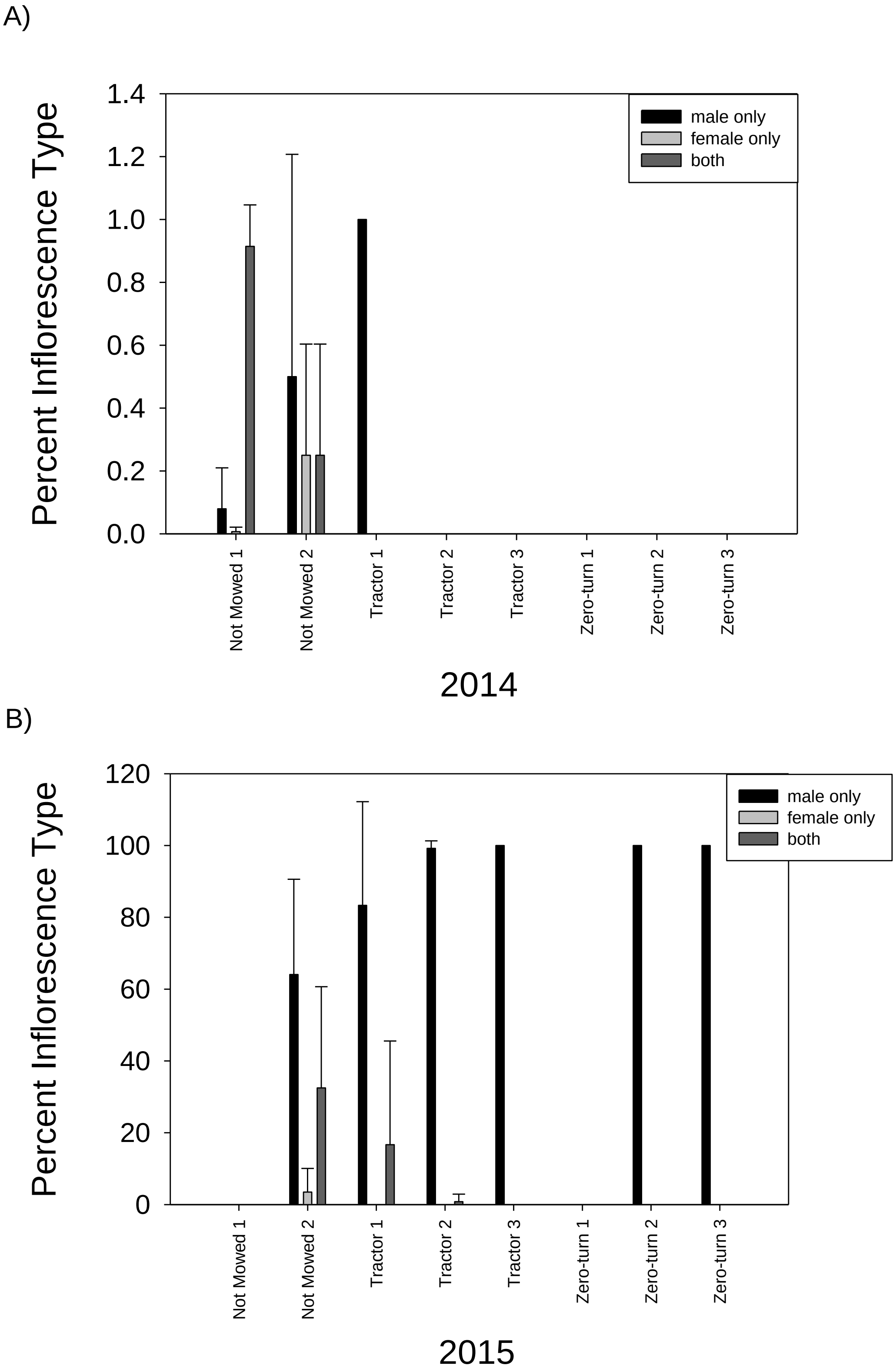


Figure 27. Mean and standard deviation of percent inflorescence type of five 0.25-m^2 plots in A) November 2014 and B) ten 1-m^2 plots in November 2015 under three mowing regimes.

Appendix
South Texas Ambrosia (*Ambrosia cheiranthifolia* A. Gray) Reintroduction Protocol

Introduction

South Texas Ambrosia (*Ambrosia cheiranthifolia* A. Gray), hereafter simply "Ambrosia," was federally listed as endangered in 1994 (59 FR 43648 43652) followed by state listing in 1997 due to it being restricted to only a few known locations in Nueces and Kleberg Counties, TX (Poole et al. 2007). Currently, there is no reintroduction protocol for this species. In response to this lack, this reintroduction protocol for South Texas Ambrosia (*Ambrosia cheiranthifolia* A. Gray) was written under Texas Parks and Wildlife Department (TPWD) Section 6 Grant Contract # 415607 based on scientific evidence collected during the course of this study and included in this final report to TPWD.

The Center for Plant Conservation (CPC) has a list of criteria that they recommend a species should meet before reintroduction is attempted (Maschinski et al. 2012):

- Distribution known and limited
- *In situ* management options inadequate for long term viability
- Threats identified
- Extinction risk high due to threats to *in situ* populations

The distribution and threats to Ambrosia are clear. Also clear is that at least at some locations long-term persistence is in doubt. Remaining unclear is the threat and viability of populations on private property that are not currently accessible. Overall, then, Ambrosia generally meets these criteria.

Species Biology

Description

Ambrosia is a herbaceous perennial in the family Asteraceae (aster or sunflower family) subtribe *Ambrosiinae*. Erect stems10-40 cm tall arise from rhizomes that may lead to considerable clonal or vegetative growth. Plants appear to remain dormant in these underground structures during harsh conditions, such as severe drought, reappearing after rainfall. This species is apparently fire-adapted as we have observed rapid resprouting after fire. Oblanceolate leaves are usually 2-7 cm long and sessile with unlobed or entire margins, which may be somewhat pinnate in young shoots. Plants are a unique grey-green color that generally makes them easy to identify in the field. This species is monoecious with a terminal male inflorescence generally 5-10cm long with flowers that open and release pollen before the axilary clusters of female flowers. Nut-like fruits, often referred to as "burs" produce a single seed or achene. Additional details can be found in Poole et al. (2007).

Current systematic treatment: This species was first collected in the area of San Fernando, Tamaulipas, Mexico in 1835 by Luis Berlandier. Asa Gray described

Ambrosia cheiranthifolia in 1859 (A. Gray in Emory 1859). The unique gray, pubescent leaves of this species make it easy to distinguish from other *Ambrosia* spp. That occur in Texas (Poole et al. 2007).

<u>Distribution and abundance</u>

Ambrosia has 27 elemental occurrences (EOs) in the Texas Natural Diversity Database (cited in USFWS 2010) in Nueces and Kleberg counties. We visited or surveyed EOs 4, 6, 16, 18, as well as some of the EOs at NAS-K during the grant period (see Table 1 and Figure 1 of this report). EO 4 appears to be extirpated, but the others are extant, including rediscovery of plants at Bishop City Park (EO 8?). Most of the populations outside NAS-K disappeared during 2007-2008 during the severe drought (Hempel and Overath 2009), but we found stems in all locations during the grant period. Stem density has varied and is declining in some locations. In addition, we reported three areas on NAS-K that experienced populations crashes in 2015. At least one of these was likely due to overmowing.

Alice Hempel (Robyn Cobb, USFWS, pers. comm.) also discovered a new occurrence in Kingsville on private property (Table 1 and Figure 1), but we were unable to gain the owners permission to survey. Populations or subpopulations at the King Ranch Training Area (EOs 19, 21-25, and 27) have been inaccessible since the mid-1990's; therefore, the status of these occurrences are currently unknown (Robyn Cobb, USFWS, pers. comm.).

<u>Ecology</u>

Ambrosia is endemic to the South Texas Coastal prairie region in both grassland mesquite savana. It is generally found at 8 to 20 meters elevation (USFWS 2010). The soils in Ambrosia locations range from heavy clay to sandy loams of the Beaumont and Victoria formations (Poole et al. 2007). Ambrosia locations in this study varied in 12 of 16 characteristics including pH, % moisture, % organic matter, and nine elements, including sodium, indicating that this species may be able to grow in a range of conditions and that the current distribution may not be indicative of optimal conditions for this species. Some of the current locations are in road right of ways that are mowed, which may reduce competition from invasive grasses such as Kleberg blustem (*Dicanthium annulatum*).

Associated species: Other native species that co-occur with Ambrosia include Texas grama (*Bouteloua rigidiseta*), buffalograss (*Buchloe dactyloides*), curly-mesquite (*Hilaria belangeri*), Texas wintergrass (*Nassella leucotricha*), littlehead gumweed (*Grindelia microcephala*), cuman ragweed (*Ambrosia psilostachya*), violet wild petunia (*Ruellia nudiflora*), coastal indigo (*Indigofera miniata*), Dakota mock vervain (*Glandularia bipinnatifida*), painted tongue (*Bouchetia erecta*), threelobe false mallow (Malvastrum *coromandelianum*), Santa Maria feverfew (*Parthenium hysterophorus*), streambed bristlegrass (*Setaria leucopila*), Drummond's clematis (*Clematis drummondii*), pyramidflower (*Melochia pyramidata*), and Texas crownbeard (*Verbesina microptera*) (Poole et al. 2007). At two sites (St. James Cemetery and US 77 right of ways) we found slender rushpea (*Hoffmanseggia tenella*) with or near Ambrosia. A major threat to Ambrosia and other coastal prairie plants in our area is the invasive grass Kleberg bluestem, which we found growing and often dominating all visited extant sites during our study.

Potential for ecotypic variation: Pollination of Ambrosia occurs as described for the congener *Ambrosia artemisiifolia* that is known to be wind-pollinated and highly outcrossing (Friedman and Barrett 2008) and we have not noticed insect pollinators visiting Ambrosia inflorescences; therefore, we conclude that this species is also wind-pollination. Wind-pollinated species tend to have high rates of outcrossing, even when self-compatible, giving Ambrosia a lower likelihood of evolving locally adapted ecotypes. However, the rhizomatous, clonal habit of Ambrosia is a confounding factor. If different clones are selected in different sites, then evolution of ecotypes may occur. Our study of genetic variation using a few microsatellite markers (recounted in this report) indicates that at least some clones are widespread; for example, one of the most common clones found at NAS-K was also found at St. James Cemetery, which is about 25 km away. Expected genomic data will shed additional light on this subject.

Reproduction and Genetics

As mentioned above, although it has not been verified genetically, Ambrosia is likely an outcrossing, wind-pollinated species. A study by Hempel in 2007 at the Robstown County Park Demonstration Site, 80% of pistallate (female) flowering heads produced fruits (USFWS 2010). However, only 41% contained filled (potentially germinable) seeds. Subsequent attempts at germination of 213 seeds, produced only one seedling. Seed viability based on the standard tetrazolium-staining method was low (14.3-25% for 10 seeds sampled from each of three individuals). Hempel also reported that the San Antonio Botanical Gardens (SABot) had successfully germinated seeds form their gardens, but details were not available (USFWS 2010). Shannon Smith, rare plant volunteer at SABot, reported that the records that are available are very unclear about this (Shannon Smith, pers. comm.). From recent observations on greenhouse plants at SABot, he has reported that fruits appear to form once female flowers open but examination of a few of mature fruits revealed no seed inside. In our own studies, we have observed no fruit production during (or after) fall flowering surveys, even at sites where female flowers were open and obviously receptive (stigmas were evident on many flowers). Our results call into question the current importance of sexual reproduction in this species.

Until our study presented in this report, no genetic studies had been conducted of Ambrosia. Although we must be cautious in our conclusions due low number of microsatellite markers we could employ, it appears that Ambrosia patches, especially larger ones, are not one clone and that several to many clones can occur in one area, which could indicate that successful sexual reproduction occurs at least occasionally. However, the fact that clones appear to be very similar genetically and the fact that we observed no seedlings even in burned plots (see Effects of prescribed burning in this report), suggests that most reproduction is asexual or vegetative. We are awaiting genomic sequence data that will clarify this issue.

Conservation

Threats

Although the destruction and fragmentation of the South Texas coastal prairie was cited as the main threat when Ambrosia was federally listed as endangered, in the most recent 5-year review (USFWS 2010) concluded that the spread of invasive grasses is now the greatest threat. Based on our own work, we concur. In all sites, Kleberg bluestem dominates or is widespread. In areas that are not regularly mowed or burned it overtops Ambrosia stems, even though these Ambrosia stems are erect, and the accumulation of dead material appears to bury Ambrosia stems over time in dense patches.

Mowing may benefit Ambrosia, or at least not harm it, by reducing competition from Kleberg bluestem plants and prevent stems from being buried. However, we have determined if mowing height is very short (ca. 3 cm), Ambrosia populations can crash. Timing of mowing may also be important to stem survival, especially in the summer months (Bush et al. 1994, Garvon 2005). Our studies show that timing of mowing can also have large impacts on reproduction by removing flowering heads.

Access to populations on currently inaccessible private lands, while not a threat, is a concern. As previously mentioned, a large number of EOs occur on at the Kingsville Training Area, which has been inaccessible for ca. 20 years. The status of these occurrences is unknown. It is also possible that currently unknown Ambrosia populations occur on other private properties in our region that contain remnant coastal prairie. These locations could also harbor unique clones or genotypes that may be important to reintroduction efforts.

A final threat we should mention is climate change. In South Texas, the climate is expected to become more variable with increased temperatures and more frequent prolonged droughts interspersed with periods of intensified precipitation (IPCC 2007). While Ambrosia can recover from drought and other adverse conditions, how long a drought period the rhizomes can survive is unknown.

Previous Propagation Work

Propgation from seeds and root cuttings: Hempel has reported that seed propagation of Ambrosia has not been successful (USFWS 2010). Smith (pers. comm.) at SABot has also reported unsuccessful seed propagation, but the records from before he took over the rare plants collection are unclear. However, SABot has been very successful in propagating Ambrosia vegetatively from cuttings. For example, they received seven root cuttings from plants at Robstown County Park in late 2002 and were able to produce over 200 plants for the Demonstration Site by mid-2006 (unpublished “History of North Nueces County Park South Texas Ambrosia project” provided by Robyn Cobb, USFWS). SABot is a CPC custodian for endangered plants and has continued to grow and propagate this species both in the greenhouse and in their gardens. Smith (pers. comm.) has reported that plants do well, but they require “thinning” on occasion or they get too “leggy.”

Demonstration site at Robstown County Park: In Fall 2006 approximately 200 Ambrosia potted plants propagated at SABot were planted in a fenced-in, prepared area at Robstown County Park, which increased to about 300 plants the next year (USFWS 2010). Ambrosia stems still occur at this site and we surveyed flowering in 2013;

however, stem density has varied, perhaps due to rainfall, but also due to lack of consistent management. Ambrosia plants in this area appear to benefit from mowing to reduce invasive grasses (pers. ob. and pers. comm. Robyn Cobb, USFWS). In our surveys the area containing Ambrosia was very similar in 2013 and 2014, but Kleberg bluestem was an obvious problem. More recently, Robyn Cobb (pers. comm.) has noted that Bernuda grass has taken hold in some areas of the site; however, plants looked healthy due to recent mowing and rains. Hempel pointed out that proper site preparation and control of exotic grasses is critical to the success of any reintroduction experiment, as are continued maintenance (Hempel and Overath 2009). We agree with this assessment. This also address with published studies on the success of reintroductions. For example, Godefroid et al. (2011) found that "reducing competition by removing surrounding plants" was a major contributor to reintroduction success.

Controlled Propagation

Collecting viable seeds from natural populations has been problematical. Hempel's study at the Demonstration site (recounted above) found extremely low, almost no germination. In addition, although their records are unclear, at least recently SABot has not been able to produce viable seeds. In addition, we have seen no fruit production in recent years in natural sites. Therefore, at least for now, seed collection from the wild and seed propagation *ex situ* do not appear to be viable options to produce material for reintroduction. However, genetic results expected soon for this study may offer guidance that will make viable seed production possible at least in controlled crosses. Overall, we can make no recommendation for seed collection from natural populations at this time, and it appears that vegetative propagation may be the best option. Given their experience and success with Ambrosia, we recommend SABot to assist in Ambrosia propagation experiments.

Refugia, Augmentation, and Reintroduction

Refugia

Currently, SABot, which is the CPC custodian for the endangered plants of South Texas, is a refugium for Ambrosia. They maintain greenhouse plants and garden plantings of this species. However, it appears that they may only have one accession and perhaps one clone/genotype (Shannon Smith, SABot, pers. comm.). Therefore, we recommend that root cuttings from plants from populations in good condition or in imminent danger be obtained for this refugium, if possible. SABot is very interested in obtaining additional accessions, in keeping with CPC recommendations (Smith, pers. comm.).

Another potential refugium is the USDA Plant Materials Center (PMC) in Kingsville. According to Hempel, in 2008 or 2009 a coastal prairie restoration experiment was planned that could have been placed at PMC (Hempel and Overath 2009). However, although a small prairie plot was prepared at PMC, it never contained Ambrosia (Shelly Maher, PMC, pers. comm.). We recommend exploring this option again and including Ambrosia in any subsequent experiment.

The experience of SABot's "unclear records" also highlights the importance of keeping detailed records. Records on plant/seed source and distribution to other refugia, seed banks, as well as any augmentation or reintroduction sites are critical.

Some of the current questions we have about, for example, reproduction results could be clarified if the number of accessions and genotypes were known.

Augmentation

Augmentation may necessary when populations become extremely small; however, because Ambrosia is known to disappear under poor conditions and reappear with good conditions, recommendations for when augmentation is necessary are problematical. One recommendation we can make is to employ methods to control invasive grasses, such as prescribed burning or mowing, before deciding augmentation is necessary. In our study, when competing invasive grasses were removed, Ambrosia rebounded quickly and stem densities were at or slightly above pre-burn levels within 6-8 weeks and flowering may have be enhanced.

In addition, the likelihood of ecotypic variation in this species is still unclear until new genetic information that is soon expected to become available. While traditional conservation practices have recommended single, related sources for augmentation and reintroduction, recent studies indicate that multiple, diverse sources may be more successful (Godefroid et al. 2011). Indeed, even when a species is composed of locally adapted ecotypes, multiple sources of material may outweigh the risks in the long term by increasing the potential for adding back selectively advantageous genes (Tom Whittam, University of Northern Arizona, symposium talk at the 2015 Ecological Society of America Annual Meeting).

Reintroduction

As stated at the outset, following CPC criteria, Ambrosia appears to be a candidate for reintroduction. However, we have grave concerns about the lack of seed material and lack of successful seed germination. Maschinski et al. (2012) recommended including some mature plants to “kick start” the population, and Godefriod et al. (2011) recommended plants rather than seeds to increase reintroduction success. However, to quickly produce variable propagules that would be desired for reintroduction (or even augmentation), successful propagation via seeds will be needed. If this is not possible, we may run the risk of not introducing enough genetic diversity in a reintroduction site for it to be successful. If reintroductions are attempted form purely vegetatively propagated material, we highly recommend using multiple diverse sources, even if genetic studies indicate local adaptation. As noted above, multiple sources appear to increase reintroduction success (Godefroid et al. 2011).

Although we are not prepared at this time to recommend specific sites for Ambrosia reintroduction, we do have some recommendations on site selection and preparation. We recommend choosing sites that are as close to possible to coastal shortgrass prairie communities or preparing sites to approximate it. We agree with Hempel that reintroducing Ambrosia in a coastal prairie community is the most likely means of success. Mechanisms for controlling invasive grasses that can be and are regularly maintained are critical; this is an important lesson from the Robstown County Park Demonstration site. We recommend growing season, prescribed burns, if possible, because some studies indicate fire is more successful at controlling invasive grasses than mowing (Havill et al. 2015, Simmons 2007). If mowing is employed, it must be timed to avoid the reproductive period.

Record Keeping Recommendations

Record keeping is crucial to conservations efforts. As we have observed through the SABot, poor records can lead to confusion and make interpreting observations difficult. We recommend the following documentation be kept for any augmentation or reintroduction:

- Existing vegetation
- Any preparation or alteration to the site before augmentation/reintroduction
- Site maps
- Numbers of each kind of propagule introduced to the site
- Source of all materials introduced to the site
- Location of that material in the field (via GPS and/or plant labels/locator stakes)
- Location of natural plants on site (in augmentations)
- Planting dates
- Prevailing site conditions

Any reintroduction should also be monitored long-term (more than the 4-year average found by Godefroid et al. 2011), including recording fate of introduced individuals and continuing methods of invasive grass control.

Summary and Recommendations

By CPC criteria, South Texas Ambrosia appears to be a candidate for reintroduction. However, success of any reintroductions will be dependent upon being able to produce a diverse array of propagules to use in any reintroduction or augmentation. This is currently problematical due to issues with the ability obtain viable seeds. Therefore, we recommend that studies of seed production and seed viability be given highest priority. In addition, the ability to control invasive grasses will be critical to any reintroduction or augmentation. Choosing remnant prairie sites or restoring sites to prairie-like conditions is also important. Finally, we recommend using multiple, diverse sources and propagating at least some plants to mature size before attempting any plantings. Any reintroduction or augmentation should also be documented and monitored as recommended above.

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Significant Deviations

Physiological Ecology

We had to abandon this part of the study. We had had some difficulties using the LiCor 6400 photosynthesis system with Ambrosia due to the size of its leaves. After several consultations with the manufacturer, we came up with some reasonable approaches. Unfortunately, before we could fully implement these strategies, we discovered a serious problem with the system that caused all measures to be incorrect. Repair of this problem required sending it to LiCor. By the time the instrument was returned it was too late to collect the planned data. This instrument is the only instrument available at TAMU-CC to make photosynthetic measurements on land plants in the field.

GPS/GIS

We had delayed this part of the study due to issues collecting soil samples needed in part of the analysis. By the time the soil data were available this fall, we were involved with the delays in the genomics sequencing (see below and completely forgot that we had not completed this part of the project. Del Mar College, where we both are now, has a very strong GIS program. We are confident that they can help us complete this part of the project by late February on the same time frame as genomics study outlined below.

Genetics

As part of our no-cost extension, we switched our genetics studies from microsatellites to genomic sequencing as described above. The methodology to do this work in a cost-effective manner in non-model organisms has only become readily available in the past few years; therefore, this is truly “cutting-edge” research. As far as we are aware, this is the first study to use these approaches on an endangered plant species. With this new methodology, ezRad, has come unforeseen challenges that have delayed the sequencing data, even though the TAMU-CC Core Lab is one of the leaders in these techniques and collaborates with and trains scientists from all over the world. We initially were told the data would be available in July, but the first challenge, which delayed library preparation and sequencing was that multiple samples had to go through several rounds of SPRI bead purification to be of the correct size and quality. The next problem the Core Facility encountered was that restriction digestion of Ambrosia was apparently more difficult than other species they have worked with and it was difficult to get fragments of the correct size; therefore, the libraries were not ready to send for sequencing until late September. The next delay was that the UT Sequencing Facility took about 6 weeks to complete the sequencing instead of taking 2-3 weeks as the Core Lab has experienced in the past, delaying sequence availability until November 6th. Quality checking of the data began immediately, which showed that too few sequences with plant DNA were returned. After pursuing several avenues and running some experiments over the next week and a half to explain the problems with the data, Dr. Chris Bird, one of the developers of ezRad and Director of the Genetics Core Lab, determined that there was a problem with their size selection procedure with Ambrosia that caused most of the correct size fragments to be excluded. Ambrosia apparently revealed in hole in their fragment validation process that has now been corrected. The Core Lab then re-prepped the fragment libraries and sent them (again) for sequencing. Dr. Bird had hoped that the sequence data would be available by mid-December, but we are still waiting. Assuming at least a 6 week wait for sequencing (as we experienced in October/November) and taking account of the ca. two weeks UT was closed in December, then we can expect the sequence data to arrive within the next two weeks. Once the data arrive, basic data analysis (i.e, enough to estimate levels of genetic diversity, but perhaps not enough to estimate clone number and size) should take 2-3 weeks. Therefore, we estimate that we can have the genomic data analyzed and this report updated by the end of February 2016.