

RICE UNIVERSITY

**The Evolutionary Ecology of Stereoisomeric
Sesquiterpene Lactones in *Xanthium strumarium***

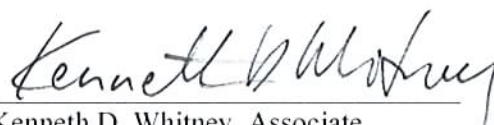
by

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ABSTRACT

The Evolutionary Ecology of Stereoisomeric
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by

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The ecological factors that maintain defensive chemical variation within and between plant species have intrigued ecologists for decades. While theory posits that polymorphisms may be maintained different forms of balancing selection, relatively few experimental studies have tested whether such balancing selection can maintain defensive chemical trait polymorphisms in nature. Further, evidence demonstrating balancing selection is rare for any trait. Here, I investigated a stereochemical trait polymorphism in *Xanthium strumarium*. This species is polymorphic with respect to the stereochemistry of the lactone ring junction of a prominent defensive compound class – the sesquiterpene lactones. Individual plants typically produce only *cis*-fused or *trans*-fused lactones across their entire suite of compounds. Sesquiterpene lactones are known to influence feeding behavior and growth rates of various herbivores, but nothing is known about the ecological implications of variation in this stereochemical trait. I first examined whether sesquiterpene lactone stereochemical variation can influence folivore feeding behavior in the laboratory. Using pure sesquiterpene lactones in controlled feeding experiments, I found that laboratory-reared grasshoppers were less deterred by the *cis*-fused compounds than the *trans*-fused compounds. I then found that these patterns extended to the field: in common gardens, plants producing *cis*-fused lactones received more damage than plants producing *trans*-fused lactones. Additionally, folivore damage was negatively correlated

with plant fitness. Taken together, these results indicate that herbivores can impose natural selection on this stereochemical trait polymorphism in nature. Finally, I found evidence that spatially variable selection leads to fitness patterns conducive to the maintenance of this polymorphism. Further, I found that the intensity of folivore damage across spatial scales predicted in which environments each morph outperformed the other, with plants producing *cis*-fused lactones achieving higher fitness than plants producing *trans*-fused lactones when herbivore pressure was low (and the reverse being true when herbivore pressure was high). This work demonstrates that relatively minor defensive chemical variation can have far-reaching impacts on the ecology and evolution of plant populations.

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LIST OF ABBREVIATIONS

CDCl₃	deuterated chloroform
CHCl₃	chloroform
HPLC	high performance liquid chromatography
HCC	University of Houston Coastal Center
KPC	Katy Prairie Conservancy
L:D	light:dark cycle
MeOH	methanol
MS	mass spectrum
nm	nanometer
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
R_f	retention factor
STL	sesquiterpene lactone
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultraviolet light
α	alpha
β	beta
μL	microLiter
μmoles	micromoles

Chapter 1

Stereochemistry Affects Sesquiterpene Lactone Bioactivity Against an Herbivorous Grasshopper

Sesquiterpene lactones are defensive compounds which protect plants against a variety of herbivores and other natural enemies. Sesquiterpene lactones from higher plants can be divided into two groups based on the stereochemistry of their lactone ring junction, either *cis*-fused or *trans*-fused. It is unclear whether and how this variation affects potentially important ecological interactions. To investigate whether stereochemical variation in sesquiterpene lactone ring junctions can influence the feeding behavior of insects, we performed controlled feeding trials with two pairs of diastereomeric sesquiterpene lactones and examined the feeding deterrent effect of each compound to the polyphagous grasshopper *Schistocerca americana*. Sesquiterpene lactone stereochemistry and concentration significantly influenced feeding behavior, with grasshoppers consuming less of the *trans*-fused compounds than the *cis*-fused compounds. To our knowledge, this is the first demonstration that sesquiterpene lactone ring junction stereochemistry influences the feeding behavior of an ecologically relevant species. Because this stereochemical trait polymorphism is widely distributed in nature, it could have substantial consequences for the ecology and evolution of large groups of plants, particularly those in the Asteraceae.

1.1 Introduction

Sesquiterpene lactones (STL) are an exceptionally diverse class of plant secondary metabolites, with close to 5000 structures elucidated to date (Harborne et al. 1999, Schmidt 2006). STL are believed to serve as defensive compounds in plants, mediating ecological interactions between plants and antagonists. They exhibit a broad range of bioactivities against different life forms and cellular processes (Rodriguez et al. 1976, Picman 1986, Schmidt 1999, 2006). STL have a broad, yet sporadic, phylogenetic distribution in plants, but are particularly diverse and abundant in the Asteraceae (Yoshioka et al. 1973). The biochemical evolution and diversification of STL in the Asteraceae, along with their putative defensive role in mediating ecological interactions, have led some to speculate that STL have contributed to the evolutionary success of this large clade (Seaman 1982, Schmidt 1999).

Given the purported ecological and evolutionary importance of sesquiterpene lactones, researchers have investigated the activity of STL against a variety of plant antagonists and competitors. Laboratory assays have indicated that STL exhibit activity against bacteria, fungi, mollusks, insects, and mammals (Rodriguez et al. 1976, Picman 1986, Schmidt 1999). They have been shown to deter feeding, reduce growth rate and weight gain, and act as developmental inhibitors (Rodriguez et al. 1976, Picman 1986, Schmidt 1999). Thus, STL are likely to be important for the survival and reproductive fitness of plants. Despite this potentially key role, we do not have a firm understanding of how even qualitative variation in STL mediates ecological interactions.

Much of the observed variation in STL activities can be explained by subtle molecular variation and the presence or absence of different functional groups (Schmidt

2006). In particular, the presence of one or more α,β -unsaturated carbonyl groups is often positively correlated with activity (Lee et al. 1971, Picman 1986, Schmidt 1999, 2006). This feature is often associated with the lactone ring that is characteristic of STL. Studies investigating quantitative structure-activity relationships (QSAR) have also found other features associated with activity, including the presence of other reactive functional groups, skeletal type, oxidation state, and partition coefficient (Schmidt 1999, 2006, Schmidt et al. 2009). Despite the identification of associations between these key structural features and STL activity, there is still a large amount of variation in activity yet to be explained.

Stereochemical variation is one understudied feature of STL that could potentially have a profound effect on activity and its ecological effects (Schmidt 2006).

Stereochemical variation commonly influences the activity of other classes of secondary metabolites and drugs (e.g. Maher and Johnson 1991, Mori 2007). Furthermore, stereochemical variation has been demonstrated to influence the activity of STL in a limited number of studies (Barbier and Benezra 1982, Streibl et al. 1983, Beekman et al. 1997, Bodensieck et al. 2011, Takeda et al. 2011). One particular aspect of STL stereochemical variation that merits further study is the stereochemistry of the lactone ring junction. The lactone ring junction is fused to the core skeleton of a STL in either a *cis* or *trans* configuration. Both *cis* and *trans* forms are found in plants, and there are numerous examples of diastereomeric compounds differing solely in the stereochemistry of their lactone ring junction (based on data presented in Budesínský and Saman 1995). *In vitro* laboratory assays with diastereomeric STL have indicated that stereochemistry can influence activity (Beekman et al. 1997, Bodensieck et al. 2011, Takeda et al. 2011).

For example, Takeda et al. (2011) found that the diastereomeric STL xanthatin and 8-epi-xanthatin differentially inhibited human breast cancer cell growth. However, the effects of stereochemical variation in STL lactone junctions has yet to be examined *in vivo*, particularly against ecologically relevant organisms, such as herbivores.

In this study, we asked the question: *does the stereochemistry of the lactone ring junction of sesquiterpene lactones influence the feeding behavior of the herbivorous grasshopper Schistocerca americana? *Xanthium strumarium* is commonly attacked by a number of folivorous insect species, including grasshoppers, in the field (Ahern Chapter 1). We first isolated four sesquiterpene lactones from the plant *Xanthium strumarium* (Asteraceae), comprising two pairs of diastereoisomers differing solely in the geometry of their lactone ring junction. We then conducted controlled, no-choice feeding experiments with the polyphagous grasshopper, *S. americana*, examining 1) whether the tested STL deterred feeding (reduced consumption) and 2) if diastereomers differed in their deterrent effect. To our knowledge, this is the first time that stereochemical variation in STL lactone junctions has been shown to influence the feeding behavior of an ecologically relevant plant antagonist.*

1.2 Methods

Test compound isolation and characterization. Aerial portions (leaves, stems, and fruits) of *Xanthium strumarium* plants were collected from two natural populations in the fall of 2010 and air dried in the laboratory. These populations were found in Liberty, Texas (30°3'23" N, 94°49'53" W), and Nixon, Texas (29°20'45" N, 97°43'39" W). *X. strumarium* is polymorphic with respect to occurrence of compounds containing a *cis*- or *trans*-fused lactone ring. Individual chemotypes of *X. strumarium* produce a suite of

sesquiterpene lactones, with a given plant generally producing either all *cis*-fused or all *trans*-fused lactone ring junctions (McMillan 1974, McMillan et al. 1975). Previous HPLC analysis indicated that all sampled individuals in one population (Liberty) produced STL with *cis*-fused lactones, while all sampled individuals in the other population (Nixon) produced *trans*-fused lactones. To prevent contamination, extraction and isolation steps for material from each collection site were performed separately, with a thorough cleaning of glassware between steps. Each step in the isolation process was performed repeatedly to acquire sufficient material before progressing to the next step. For simplicity, we detail the general isolation procedures used below.

All steps in the isolation process were monitored via TLC, using chloroform:acetone 9:1 as the solvent, and visualized using a standard vanillin spray reagent. The vanillin spray reagent reacts with many sesquiterpene lactones to produce characteristic color patterns. Under the conditions described, xanthatin and 8-epi-xanthatin developed into a characteristic bright red spot with an R_f of 0.48 after heating, while xanthinosin and tomentosin develop to a bright blue with an R_f of 0.52 after heating. Structures of the four compounds are presented in Figure 1.1.

Plant material was extracted three times by immersing in methylene chloride for 30 minutes. The combined extracts were vacuum filtered through Whatman #1 filter paper to remove solid particulate matter and subsequently concentrated by rotary evaporation. Next, we separated this extract into crude fractions via dry column vacuum chromatography (Harwood 1985). For each extraction, we used a 350 ml glass fritted Buchner funnel packed with ~5cm of silica gel 60 (32-63 μ m). This column was equilibrated with the elution solvent, then run using a stepwise gradient of hexanes:ethyl

acetate, starting at 70:30 and gradually increasing to 50:50, collecting 150 mL fractions. Because our extract was not sufficiently soluble in the starting elution solvent mixture, we dry-loaded the column by dissolving the extract in chloroform, mixing with dry silica gel 60 (32-63 μm), drying this down in a rotary evaporator, and applying the free flowing silica to the top of the packed column. Fractions rich in the target sesquiterpene lactones were pooled and concentrated with a rotary evaporator. Next, compounds were separated via flash chromatography in a 1.5 cm ID column using a stepwise gradient of chloroform:methanol, starting at 99.8:0.2 and gradually increasing to 98:2, collecting 9 mL fractions. Pure fractions were pooled and concentrated with a rotary evaporator, while mixed fractions were re-chromatographed.

Structural Identification. All sesquiterpene lactones were identified by ^1H and 2D NOESY experiments. Analyses were performed on a Varian Inova 600 w/HCN cold probe at 22°C in CDCl_3 with 0.1% TMS as an internal standard. Compounds were identified by comparison with published ^1H NMR data of sesquiterpene lactones isolated from *Xanthium strumarium*. For each compound, the references were: tomentosin (McMillan et al. 1975, Bohlmann et al. 1978, Lanzetta et al. 1991, Park et al. 2001, Kim et al. 2004), xanthinosin (McMillan et al. 1975, Bohlmann et al. 1978, Ahn et al. 1995, Ramirez-Erosa et al. 2007), 8-epi-xanthain (McMillan et al. 1975, Ahn et al. 1995, Yokotani-Tomita et al. 1997, Kummer et al. 2006, Matsuo et al. 2010), xanthatin (McMillan et al. 1975, Ahn et al. 1995, Ramirez-Erosa et al. 2007, Matsuo et al. 2010). All compounds were estimated at >90% purity based on ^1H spectra. In order to verify the stereochemical assignment of the lactone junction for each compound, we performed NOESY experiments using the same instrumentation described above. We made

stereochemical assignments based on NOE's between H-8 and adjacent protons (Figure 1.2).

Grasshoppers. *Schistocerca americana* (Drury) is a polyphagous grasshopper, feeding on a wide range of plants, including many Asteraceae (Kuitert and Connin 1952, Otte 1975). It occurs naturally throughout the southeastern United States (Harvey 1981). All grasshoppers used in experiments were reared from eggs from a standing laboratory colony maintained since 2006 by S. Behmer, Texas A&M University (Boswell et al. 2008). Eggs were hatched and nymphs maintained in an insectary in Bioquip cages (61 x 61 x 61cm) at 31°C, and photophase L:D 15:9. Newly hatched nymphs were fed *ad libetum* on a mixed diet of fresh wheatgrass seedlings and dry wheat germ throughout their entire developmental period. All experiments were conducted with 1-2 day old 6th instar grasshoppers.

Grasshopper stock suffered from persistent infection with the protozoan parasite *Malamoeba locustae*. This parasite is common in laboratory cultures of grasshoppers, and causes reduced growth rate and mortality (Hinks and Erlandson 1994). In order to reduce parasite load in infected grasshoppers, we treated wheatgrass seedlings and wheat germ with a 5% aqueous solution of the antibiotic sulfamethazine sodium salt, beginning immediately after egg hatch. This antibiotic has been shown to reduce or eliminate the persistence of *Malamoeba locustae* in laboratory culture (Hinks and Erlandson 1994). In order to minimize chances of interactions between this antibiotic and test compounds, grasshoppers were removed daily as they molted to the 5th instar, transferred to new rearing cages, where their diet was switched to untreated (antibiotic-free) wheatgrass and wheat germ. Subsequently, as grasshoppers molted to the 6th instar, they were again

removed daily and transferred to new rearing cages. Thus, all grasshoppers developed through an entire nymphal instar without antibiotics prior to their use in experiments. Preliminary trials with infected (untreated) grasshoppers gave similar results to those presented here.

Experimental protocol. To assess the influence of lactone junction stereochemistry on feeding deterrence in *S. americana*, we conducted no-choice feeding experiments with individual STL applied to glass microfiber discs (Whatman GF/A, 2.1 cm diameter). We chose to use microfiber discs, because a) their mass and area is very uniform, and b) they are a neutral substrate, reducing the potential of interactions between target STL and other metabolites. Such discs have been widely used in grasshopper feeding trials (e.g. Chapman et al. 1988, Cottee et al. 1988, Behmer and Elias 1999).

Each compound was tested across a range of five concentrations, constituting 1, 2, 3, 4, or 5% dry weight of the disc. This corresponds to a range of ~ 0.759 - 3.796 μmols per disc for xanthatin and 8-epi-xanthatin (MW 246.30), and ~ 0.753 - 3.765 μmols per disc for xanthinosin and tomentosin (MW 248.32). These concentrations are biologically relevant; in *Xanthium strumarium* individual STL compounds have been measured in excess of 2% dry weight (Winters 1969) and total STL content has been measured at over 3% dry weight (Chavez 1973). Further, total STL content has been measured at over 5% in other Asteraceae species (Rodriguez et al. 1976). Each pair of trials consisted of 11 treatments; five concentrations of the *cis*-fused compound, 5 concentrations of the *trans*-fused compound, along with one control. The control was used to measure baseline consumption in the absence of sesquiterpene lactones.

To stimulate feeding on each disc, we applied sucrose to each disc at a rate of 2.5% dry weight. This rate has been shown to act as a phagostimulant in similar experiments examining grasshopper feeding (Behmer and Joern 1993). To prepare disks for feeding assays, we first coated each disk with 100 μL of aqueous sucrose solution and allowed them to air dry overnight. On the morning of the trial, discs were coated with 120 μL of chloroform solution containing the test compounds and allowed to air dry. These volumes were deemed sufficient to completely saturate a disc with each solvent. For the control treatment, we applied 120 μL of pure chloroform to the sucrose-treated disc.

Each test arena consisted of a 17 x 12 x 6.5 cm (L x W x H) clear plastic box with ventilation holes in the top. The test filter disc was mounted on an inverted pushpin placed 6.5 and 4 cm from the sides of the box so that the disc was readily accessible to the grasshopper. The other side of each box contained a water source (plastic portion cup with a cotton wick), and a perch made of metal hardware cloth. All boxes were arranged on a table in a testing room, illuminated with overhead incandescent light and maintained at 31°C. We erected cardboard partitions between boxes to prevent visual interactions between neighboring grasshoppers.

All trials were conducted with 1-2 day old 6th instar nymphs. On the morning of each trial, all food was removed from cages at 8:30 am, and grasshoppers were starved for two hours. Grasshoppers were then introduced to the test arenas at 10:30 am, and removed 7 h later at 5:30 pm. Discs with no detectible damage were assumed to have been associated with highly infected and unhealthy grasshoppers and were excluded from the analysis, but preliminary analyses including these data yielded qualitatively similar results. After the completion of each trial, grasshoppers were removed and weighed

individually so that grasshopper weight could be included as a covariate in the statistical models (below). Discs were weighed after each trial and compared to the initial weight of the disc to calculate the percentage mass removed. Because of space limitations, we examined feeding responses to each pair of diastereomers in separate experiments, and conducted each trial over a series of days. Because of this constraint, each pair of diastereomers was examined in separate statistical models (below), and trial day was incorporated into each model. This constraint prevented us from statistically testing for differences between pairs, as trial date was confounded with the identity of the tested pair.

Statistical Analysis. In each pair of trials, grasshoppers consumed less than 100% of the control discs (those without STL test compounds), and the average percentage of control discs consumed differed between trials. To simplify the interpretation of results, we divided the percentage of each disc consumed by the average percentage consumption of control discs (78.4% for tomentosin: xanthinosin, 71.6% for 8-epi-xanthatin: xanthatin) to estimate the percentage of control consumption. Although parameter estimates differed, results from analyses of raw data and these standardized data were identical.

Statistical analyses were performed using Proc Mixed in SAS (version 9.3, SAS institute). For each pair of compounds we fit a mixed ANCOVA model. Percentage of control consumption was the response variable, while stereochemistry (either *cis* or *trans*), concentration, and trial date were predictor variables. Grasshopper weight was not included in the model after preliminary analyses determined that it did not improve model fit. Stereochemistry was treated as a categorical fixed effect, concentration as a

continuous fixed effect, and trial date was treated as a categorical random effect.

Interactive effects between stereochemistry and concentration were not included in the final model based on both significance tests and AIC, which indicated that dosage-dependency did not differ between *cis*- and *trans*-fused compounds. We compared the least squares means of the response (percentage of control consumption) for each compound to determine whether there was an overall effect of stereochemistry.

1.3 Results

All tested sesquiterpene lactones reduced feeding consumption by *S. americana*. In trials with both pairs of compounds, both sesquiterpene lactone concentration and stereochemistry significantly influenced consumption by grasshoppers (Figure 1.3). For the diastereomeric pair tomentosin:xanthinosin, percentage consumption was reduced as concentration increased ($F_{1,189} = 103.06$, $P < 0.0001$), and grasshoppers consumed more from discs coated with tomentosin (*cis*) than xanthinosin (*trans*) ($F_{1,189} = 25.58$, $P < 0.0001$). Similarly, for the pair 8-epi-xanthatin:xanthatin, percentage consumption was reduced as concentration increased ($F_{1,198} = 59.28$, $P < 0.0001$), and grasshoppers consumed more from discs coated with 8-epi-xanthatin (*cis*) than xanthatin (*trans*) ($F_{1,198} = 10.72$, $P = 0.0013$). The absence of significant interactive effects between stereochemistry and concentration in preliminary analyses indicated that the dose-dependency of the response of grasshoppers to concentration was similar for *cis*- and *trans*-fused sesquiterpene lactones.

1.4 Discussion

In this study, we have demonstrated that sesquiterpene lactone ring junction stereochemistry influences the feeding behavior of the polyphagous grasshopper,

Schistocerca americana. In two independent trials with pairs of diastereomeric compounds, we found that while all four STL tested reduced grasshopper feeding, those with *trans*-fused lactone junctions reduced grasshopper consumption 31.8% more than those with *cis*-fused lactone junctions. Importantly, this is the first empirical evidence that naturally-occurring variation in sesquiterpene lactone stereochemistry influences the feeding behavior of an ecologically relevant herbivore (see also discussion below of Streibl et al. 1983) and thus influences plant resistance to herbivores. These findings indicate that stereochemical variation in sesquiterpene lactones may be an important, understudied factor mediating plant-herbivore interactions.

Possible mechanisms of bioactivity. Stereochemical variation in the lactone ring junction is likely to influence the biological activities of STL via ring strain-based activity and/or target-based activity. The lactone ring of STL often possesses an α,β -unsaturated carbonyl group, a feature commonly associated with bioactivity (Lee et al. 1971, Picman 1986, Schmidt 1999). Stereochemical variation in this ring affects the overall molecular geometry of the compound, potentially influencing the ways in which reactive functional groups interact with target sites (Beekman et al. 1997).

Importance of stereochemical variation in the evolutionary ecology of STL. To our knowledge, only one other study has documented that stereochemical variation in sesquiterpene lactones can influence insect feeding deterrence. Streibl et al (1983) examined the enantiomeric STL's isoalantolactone and *ent*-isoalantolactone for deterrent activity against three grain feeding beetles. They found that isoalantolactone was significantly more deterrent to adult *Tribolium confusum* beetles than its enantiomer *ent*-isoalantolactone, but the pattern was reversed for larvae of *Trogoderma granarium*.

These findings are interesting, yet their ecological relevance is questionable because one of the test compounds (*ent*-isoalantolactone) does not naturally occur in higher plants, the host species of the tested beetles. However, experiments on a number of different plant secondary metabolites have demonstrated that stereochemical variation can influence the feeding behavior of insects. For example, the diastereomeric alkaloids quinine and quinidine have been shown to differentially affect the growth and survival of *Spodoptera littoralis* caterpillars (Krug and Proksch 1993). Similarly, the enantiomeric monoterpenes (*R*)-(+)-pulegone and (*S*)-(-)-pulegone have been demonstrated to differentially deter feeding by Colorado potato beetles and aphids (Szczepanik et al. 2005, Dancewicz et al. 2008).

Despite the expectation that variation in plant defensive chemical traits is maintained by different physiological or ecological costs and benefits (Simms and Rausher 1987, Strauss et al. 2002), we currently have no evidence that *cis*-fused STL are less costly to produce than *trans*-fused STL. Our current understanding of STL biosynthesis indicates that the lactone ring junction is formed by the stereospecific enzymatic hydroxylation of the precursor compound. Ikezawa et al. (2011) demonstrated that homologous P450 monooxygenases from lettuce and sunflower are responsible for the stereospecific formation of *cis* and *trans* fused STL found in each respective species. Based on the assumption that the different homologs responsible for lactone ring formation in *X. strumarium* do not have different energetic requirements, we have no *a priori* expectation for differences in the energetic production costs of *cis*- and *trans*-fused STL. Indeed, field observations under experimentally reduced herbivory (insecticide treatment) revealed no significant differences in fitness between *X. strumarium* bearing

cis- versus *trans*-fused STL (Ahern Chapter 1). These observations, in conjunction with the effects on feeding behavior reported here, have important implications for the evolutionary ecology of STL in plants. If there are no energetic differences in the costs associated with producing *cis* and *trans*-fused STL, but there are benefits to producing *trans*-fused STL, one would expect plants producing *cis*-fused STL to be at a selective disadvantage. However, the persistence of both *cis*- and *trans*-fused STL in nature suggests that each form may confer a selective advantage for plants in some situations. One likely possibility is that *cis* and *trans*-fused STL confer higher resistance to different herbivore species. Although there is no evidence yet for this hypothesis for *X. strumarium*, evidence from enantiomeric STL indicates this is a possibility (Streibl et al. 1983).

Further examination of the ecological effects of STL could also inform understanding of the macroevolutionary patterns of plant-herbivore interactions across broad plant clades. Sesquiterpene lactones are widely distributed phylogenetically but have an exceptionally high diversity in the Asteraceae. In a preliminary compilation of STL stereochemistry reported in the literature, we found both *cis*-fused and *trans*-fused STL are distributed across the Asteraceae; of the 192 genera in our database, 168 genera produced *trans*-fused STL while 69 produced *cis*-fused STL (Ahern, unpublished data). Further, 45 of these genera (~23%) are known to produce both *cis*- and *trans*-fused STL (rather than just a single form). A better understanding of how STL stereochemical variation influences ecological interactions could help elucidate broader evolutionary patterns in the Asteraceae, the largest plant family. For instance, it would be intriguing to

investigate whether plants producing *cis* or *trans*-fused STL commonly host different herbivore groups.

In conclusion, stereochemical variation in sesquiterpene lactones likely plays an important, yet unrecognized role, in mediating plant-herbivore interactions. We advocate new research into the ecological consequences of sesquiterpene lactone stereochemical variation. In particular, the generality of the pattern reported in this paper, in which *trans*-fused STL conferred more resistance than *cis*-fused STL, should be addressed with more studies. Whether the patterns observed here are consistent across a range of herbivore species or across a range of other diastereomeric STL remains to be examined.

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1.7 Figures

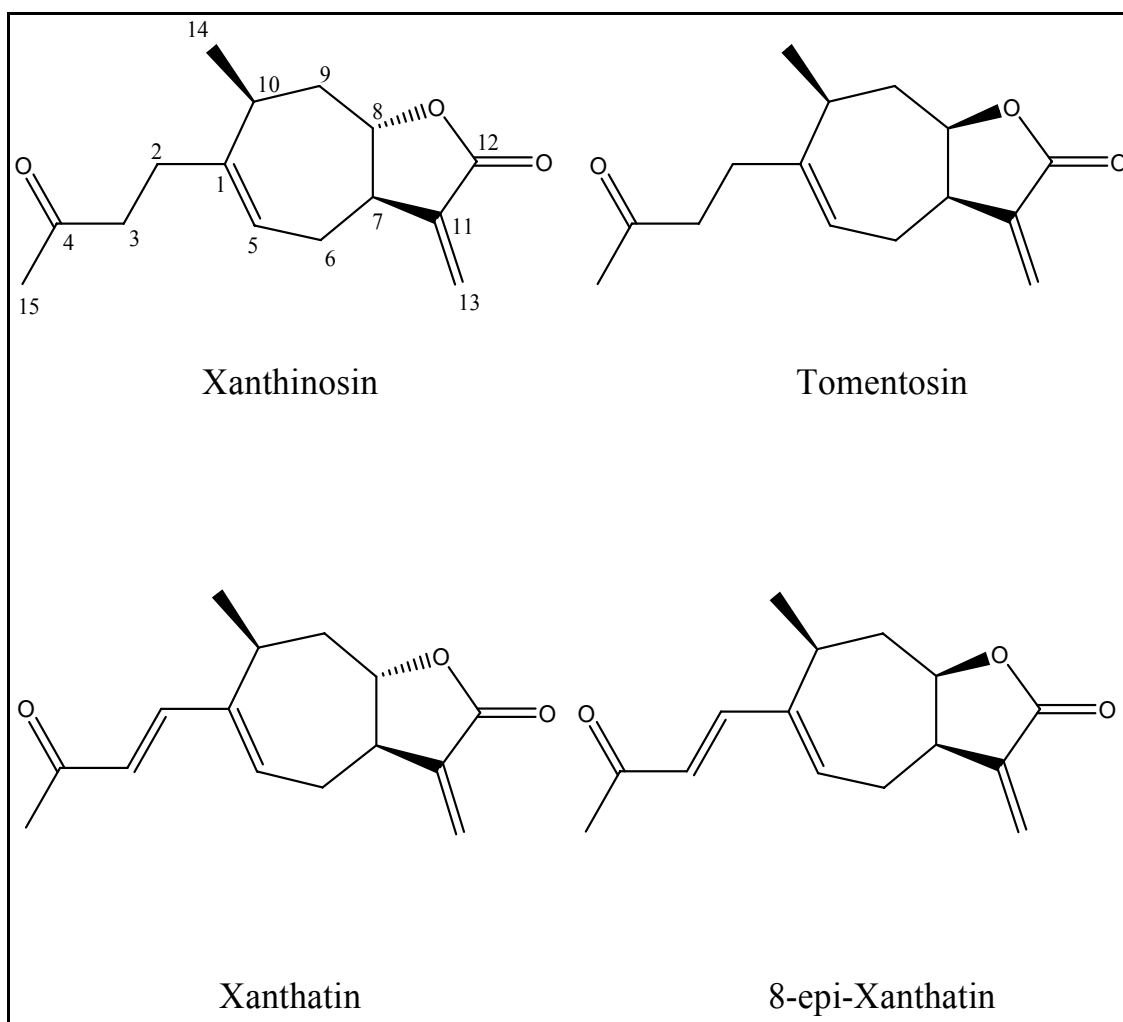


Figure 1.1. Structures of sesquiterpene lactones utilized in this study. The *trans*-fused sesquiterpene lactones, xanthinosin and xanthatin, are illustrated on the left while the *cis*-fused sesquiterpene lactones, tomentosin and 8-epi-xanthatin, are illustrated on the right. The two diastereomer pairs differ solely in the stereochemistry of their lactone ring junctions at carbon 8.

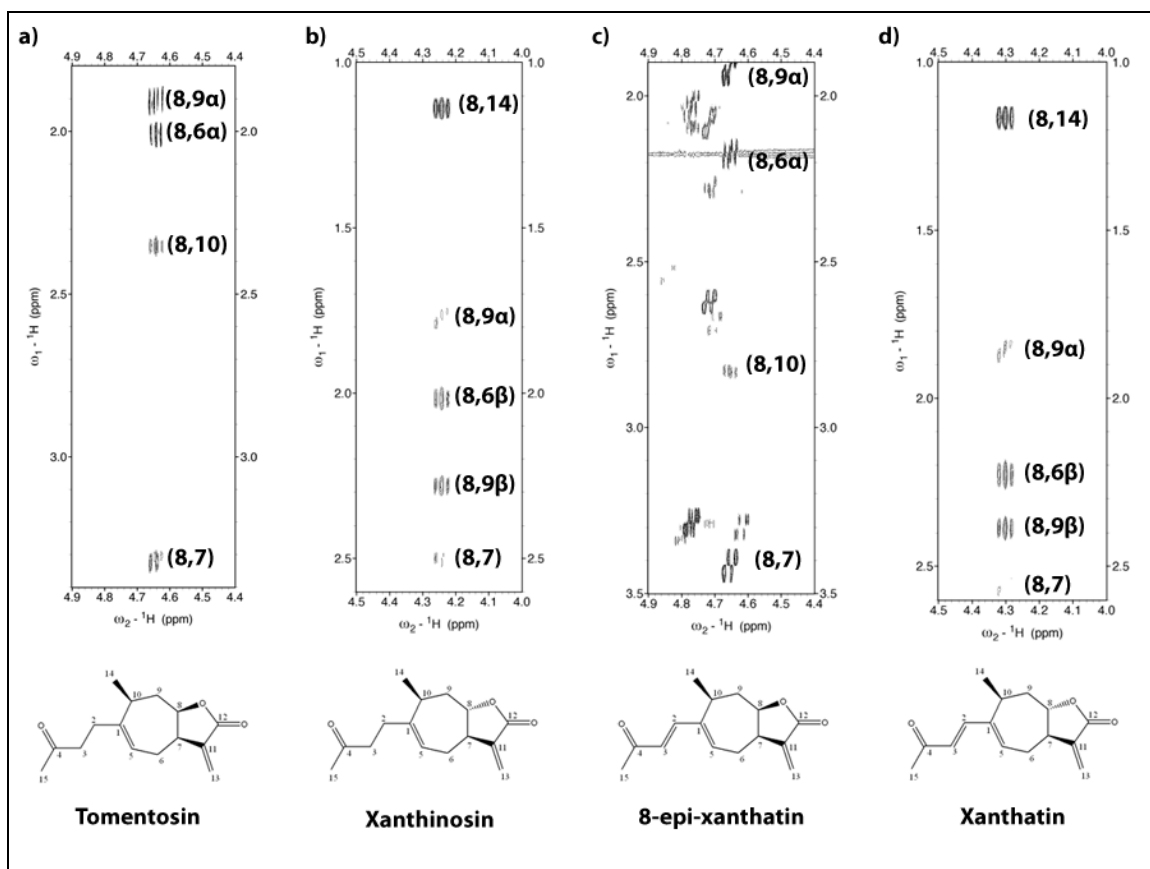


Figure 1.2. NOESY experiments verified the stereochemical assignments of the four different compounds. *Cis*-fused compounds (tomentosin and 8-epi-xanthatin) had strong NOE's between the epimeric proton 8 and protons 6 α , 7, 9 α , and 10. *Trans*-fused compounds (xanthinosin and xanthatin) had strong NOE's between the epimeric proton 8 and protons 6 β , 9, and 14 along with weak NOE's with protons 7 and 9 α .

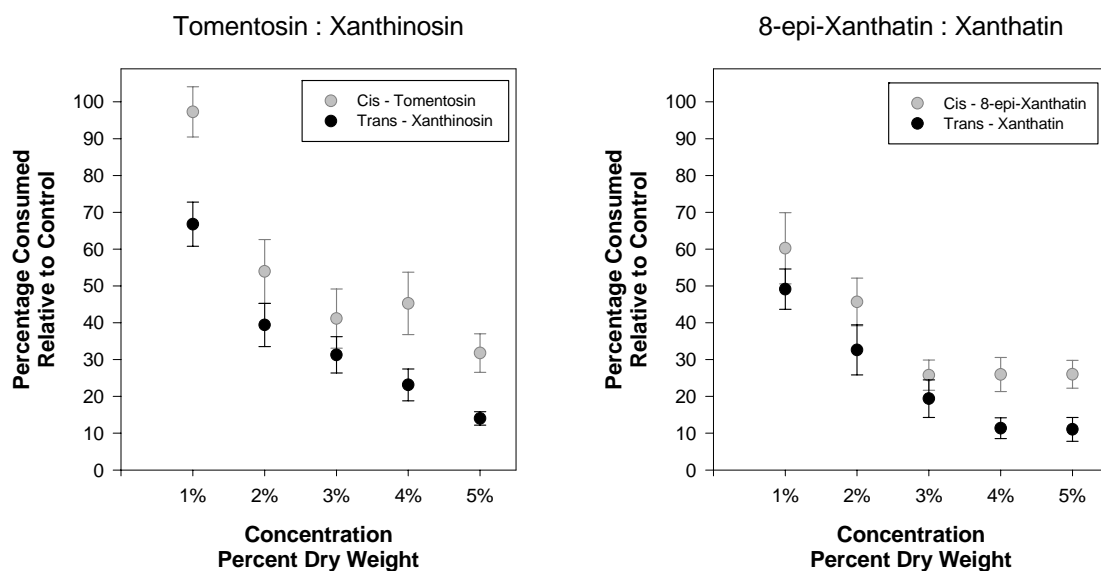


Figure 1.3 Percentage consumption of discs by *Schistocerca americana* grasshoppers in response to sesquiterpene lactone stereochemistry and percentage dry weight concentration for two diastereomeric pairs. Consumption measures for test discs were relativized to the consumption of control discs. Least squares means and standard errors are presented for each treatment combination. *Cis*-fused STL are presented in gray, *trans*-fused STL are presented in black. In both trials, both stereochemistry and concentration significantly influenced feeding consumption by grasshoppers. Least squares mean consumption of each compound was: tomentosin, 57.74%; xanthinosin, 38.94%; 8-epi-xanthatin, 36.28%; xanthatin, 24.97%.

Chapter 2

Sesquiterpene lactone stereochemistry influences herbivore resistance and plant fitness

Stereochemical variation is widely known to influence the bioactivity of compounds in the context of pharmacology and pesticide science, but our understanding of its importance in mediating plant-herbivore interactions is limited, particularly in field settings. Similarly, sesquiterpene lactones are a broadly distributed class of putative defensive compounds, but little is known about their activities in the field. We used natural variation in sesquiterpene lactones of the common cocklebur, *Xanthium strumarium* (Asteraceae), and a series of common garden experiments to examine relationships between stereochemical variation, herbivore damage, and plant fitness. We found evidence of substantial heritable variation in herbivore resistance between source populations. These source populations could be classified into two chemotypes based on the stereochemistry of the sesquiterpene lactone ring junction: plants producing *cis*-fused sesquiterpene lactones and plants producing *trans*-fused sesquiterpene lactones. Across both years and sites, the former experienced significantly higher levels of damage from leaf chewing insects than the latter. Experiments manipulating herbivore damage above and below ambient levels found herbivore damage was negatively correlated with plant fitness. Together, these results indicate that subtle differences in stereochemistry may be a major, but little appreciated, determinant of the protective role of secondary metabolites in plants.

2.1 Introduction

Understanding the connections between plant secondary metabolites and their ecological and evolutionary consequences has been a goal of chemical ecologists for more than half a century (Fraenkel 1959, Ehrlich and Raven 1964, Berenbaum and Zangerl 2008, Agrawal 2011). Many plant secondary metabolite classes have been shown to influence herbivore resistance and subsequent plant fitness in the field, including glucosinolates (Mauricio 2000, Bidart-Bouzat and Kliebenstein 2008), furanocoumarins (Berenbaum et al. 1986) cardenolides (Agrawal 2005), and alkaloids (Shonle and Bergelson 2000, Macel and Klinkhamer 2010). From these and other studies, it is clear that within a particular class of secondary metabolites, the structure of different compounds influences herbivores and other natural enemies. For example, in aliphatic glucosinolates, variation in side-chain elongation and modification has been associated with variation in activity against different insect species (Giamoustaris and Mithen 1995, Mithen 2001). However, in comparison to this focus on the ecological effects of structural variation, very little attention has been paid to the ecological effects of stereochemical variation. Stereoisomers are compounds that have the same molecular formulas and bond connections, but differ in the three-dimensional orientations of their atoms in space. Stereochemical variation is extremely common in plant secondary metabolites, occurring e.g. in terpenoids, alkaloids, flavonoids, glucosinolates, and coumarins (based on examination of data in Buckingham 2011).

Despite the fact that they share an identical structure, a pair of stereoisomers may differ in biological activities because differences in three-dimensional shape can lead to altered interactions with other molecules (Kalsi 2009). This fact is widely appreciated in the fields of pesticide science, pheromone science, and pharmacology (Maher and Johnson 1991, Williams 1996, Nguyun et al. 2005, Mori 2007, Nunez et al. 2009, Garrison 2011). In some cases, individual isomers have identical bioactivities, but in other cases different isomers can drastically differ in bioactivity (Patel & Hutt 2004). Pesticide science is rife with examples of the differential effects of stereoisomers. For example, the (*S,S*) isomer of the insecticide fenvalerate (a synthetic pyrethroid) has been shown to be two to three orders of magnitude more toxic than its other isomers (Bradbury et al. 1987). Synthesizing and characterizing individual stereoisomers with increased potency also led to the development of deltamethrin (among many others), changing the course of pest management (Elliott et al. 1974, Casida 2010).

Stereochemical variation in naturally occurring plant defensive chemicals has also been shown to influence bioactivity. Laboratory bioassays have shown that stereochemical variation in a variety of plant secondary metabolites can influence the feeding behavior and dietary preference of insects, including studies on monoterpenes (e.g. Peterson et al. 2002, Tooker and Hanks 2004, Dancewicz et al. 2008), sesquiterpenes (e.g. Collins et al. 2000), polyphenols (e.g. Stipanovic et al. 2006), and alkaloids (e.g. Krug and Proksch 1993). We have recently shown that stereochemical variation in sesquiterpene lactones influences grasshopper feeding deterrence (Ahern, Chapter 1). These laboratory bioassays clearly demonstrate that stereochemical variation

in plant defensive chemicals can influence insect feeding behavior and dietary preference, and therefore has the potential to be relevant in a field setting.

However, although laboratory bioassays provide invaluable information about herbivore feeding preference and performance, field studies are needed to demonstrate the influence of stereochemical variation on the ecology and evolution of plant chemical defense. In field settings, plants interact with a diversity of herbivore species, and it is the net effect of these species that determines the selective impact of herbivores on plant defense (Iwao and Rausher 1997). Different herbivore species can often have markedly different responses to plant defensive chemicals (e.g. Iason et al. 2011), generating cases where apparent benefits of plant chemical defenses demonstrated in laboratory bioassays conflict with observed patterns of herbivore damage in field settings (e.g. Burnett et al 1977a). Despite the acknowledged importance of quantifying the combined impact of multiple herbivores on plant defensive chemical traits, field experiments examining how stereochemical variation in plant defensive chemicals influences ecological interactions are, to our knowledge, currently lacking.

Here, we investigated the link between stereochemical variation in sesquiterpene lactones and herbivore resistance in the field, using *Xanthium strumarium* L. (Asteraceae) as a study system. *X. strumarium* is polymorphic in sesquiterpene lactone ring junction stereochemistry, with an individual plant typically producing only *cis*- or only *trans*-fused lactones (Figure 2.1) across its entire suite of compounds (McMillan et al. 1975b). Specifically, we asked the following questions: **1)** Is there heritable variation in herbivore resistance in *Xanthium strumarium*? **2)** Is sesquiterpene lactone stereochemistry associated with herbivore resistance? and, **3)** Does herbivore damage

reduce plant fitness (i.e., is herbivore resistance under natural selection)? To address these questions, we made a large number of *X. strumarium* seed collections from North America and Asia and utilized a common garden approach in southeast Texas. In 2008, we established a common garden to investigate whether there was heritable variation in herbivore resistance and whether stereochemistry influenced herbivore resistance. In 2009, we established two common gardens where we manipulated leaf damage above and below ambient levels to address whether damage influenced plant fitness. Using the control (ambient herbivory) plants in the 2009 gardens, we again examined variation in resistance and the relationship between stereochemistry and resistance. To our knowledge, our work represents only the second study to examine STL as defensive compounds in the field (as opposed to laboratory settings; see also Burnett et al. 1977a), the first to document herbivore-mediated natural selection on STL variation, and the first to investigate the ecological significance of the stereochemistry of the lactone ring junction. Our results demonstrate that STL stereochemistry can have large impacts on resistance and on plant fitness, suggesting a dynamic evolutionary history within the Asteraceae and other plant families.

2.2 Materials and Methods

Study chemicals. Sesquiterpene lactones (hereafter STL) are a diverse class of terpenoids with close to 5,000 different structures elucidated (Harborne et al. 1999, Schmidt 2006). They are broadly distributed, occurring in the fungi (Fischer et al. 1979), liverworts (Asakawa 2004), and angiosperms (Fischer et al. 1979). They are particularly diverse in the Asteraceae, with over 3000 described structures (Fischer et al. 1979, Seaman 1982). Laboratory trials have indicated that STL are biologically active against

bacterium, fungi, invertebrates, and vertebrates (Rodriguez et al. 1976, Picman 1986, Schmidt 1999). This bioactivity is most commonly linked to α,β -unsaturated carbonyl groups (Schmidt 1999). These reactive groups bind to biological macromolecules via a Michael-type addition reaction, and often result in the deactivation of enzymes (Picman 1986, Schmidt 1999). Despite the near ubiquity of this active site in STL, there is a great deal of unexplained variation in their bioactivities (Schmidt 1999). Furthermore, we know little about how STL variation influences ecological interactions in the field, as only a single study system has been examined (Burnett et al. 1977a, b, Mabry et al. 1977). In these studies the authors examined plant resistance to insect and mammalian herbivores in three related *Vernonia* (Asteraceae) species, one of which does not produce STL. Species producing STL were more resistant to mammalian herbivores, but less resistant to insect herbivores, than the STL-deficient species. Note that the result for insect resistance is not consistent with laboratory trials showing a deterrent effect of STL on insects (Burnett et al. 1977a), underscoring that we have much to learn about the roles of STL in nature.

A key feature of STL is the presence of stereochemical variation in the lactone ring junction; the lactone ring can be fused to the remaining skeleton in either a *cis* or *trans* configuration (Figure 2.1). Hereafter we will refer to these as *cis*- and *trans*-fused STL. Both *cis*- and *trans*-fused forms are found in angiosperms (based on data presented in Seaman 1982, Budesínský and Saman 1995). Within the Asteraceae, *trans*-fused forms appear to be more common: a preliminary compilation of records from the literature (Ahern et al., unpublished) has documented *cis*-fused forms of STL in 69 genera (in 23 tribes) and *trans*-fused forms in 168 genera (in 39 tribes) with overlap of

cis- and *trans*-fused lactones in ~23% of genera and ~26% of tribes. While this variation could be selectively neutral (i.e., it may have no effect on bioactivity), the presence of this extensive stereochemical variation and its unequal representation across the Asteraceae suggests a potential signal of natural selection: for example, it may be that each stereoisomer provides high fitness in different environments or against different plant enemies. Using pure extracts of two pairs of STL, we have recently shown that stereochemical variation influences feeding deterrence to the polyphagous grasshopper *Schistocerca americana* in laboratory settings (Ahern, Chapter 1). However, the importance of sesquiterpene lactone stereochemical variation in plant populations under field conditions has yet to be examined.

Study Species. *Xanthium strumarium*, the common cocklebur, is a cosmopolitan annual that inhabits ruderal habitats (Weaver and Lechowicz 1983). It is a monoecious annual with spatially separated male and female flowers and, though wind pollinated, it is primarily an inbreeder because male flowers develop above female flowers, raining pollen directly onto them (Löve and Dansereau 1959, Weaver and Lechowicz 1983). This breeding system, along with periodic outcrossing, results in differentiated monomorphic populations, with much of the phenotypic variation found among, rather than within, populations. Variation among populations is so extensive that >50 species epithets were previously recognized in different floras in North America (Löve and Dansereau 1959). However, most current treatments recognize only a single species e.g. Flora of North America Editorial Committee (1993+). Female flowers produce indehiscent two-seeded fruits (burs) that are dispersed by gravity, water, or animals via hooked spines on the bur surface (Weaver and Lechowicz 1983).

The prominent defensive compounds in *X. strumarium* are sesquiterpene lactones, occurring in concentrations in excess of 3% dry weight (Chavez 1973). Only 4-6 individual compounds have generally been reported in a given population of plants, although >40 different STL have been isolated from *X. strumarium*, indicating substantial intraspecific variation (McMillan et al. 1975b, Zhang et al. 2001). A key component of this diversity is the stereochemistry of the lactone ring junction, which can be either *cis*- or *trans*-fused (Figure 2.1). *X. strumarium* is polymorphic, in that within the species as a whole both *cis*- and *trans*-fused lactones are found, although most natural populations are monomorphic (all of the plants produce either all *cis*-fused or all *trans*-fused STL) (McMillan et al. 1975a, McMillan et al. 1975b). It has been hypothesized that this pattern is due to allelic variation in the enzyme responsible forming the lactone ring junction (J. W. de Kraker, pers. comm.; D. K. Ro, pers. comm.). This enzyme, a cytochrome p450 hydroxylase, is responsible for stereospecific ring formation in other plant species (de Kraker et al. 2002, Ikezawa et al. 2011). This reaction occurs early in the biosynthesis of sesquiterpene lactones, which subsequently undergo substantial modification, leading to numerous end products (Fischer et al. 1979).

STL from *Xanthium* have been examined for bioactivity against a wide range of organisms including insects, fungi, protozoa, bacteria, and mammalian cells (Little et al. 1950, Kawazu et al. 1979, Roussakis et al. 1994, Lavault et al. 2005). Additionally, stereochemical variation in STL from *X. strumarium* has been demonstrated to influence feeding behavior of grasshoppers: in bioassays using pure extracts of two diastereomeric pairs of STL, the *trans* form was significantly more deterrent than the *cis* form (Ahern Chapter 1). However, all studies to date have been laboratory bioassays, and the

implications of stereochemical variation of *X. strumarium* STL in field settings, and their fitness consequences for the plant have yet to be examined.

Plant Material. Mature burs were collected in the field in the fall of 2007 and 2008 from 19 sites in North America and Asia (Table 2.1). Individual seeds were excised from burs and germinated on moist tissue paper in the laboratory. Germinated seeds were transplanted into 3.8 x 10 cm Jiffy peat pellets, and grown in the greenhouse for approximately 2 weeks prior to planting in the field.

Chemotyping of Plant Material. In order to isolate a reference STL, bulk material was collected from the AUS population (see Table 2.1) in the fall of 2007. Dried ground leaf material (1.5 kg) was soaked in chloroform and the extract dried *in vacuo*. The resulting residue (~110 grams) was separated by column chromatography with silica gel starting with hexanes, followed by CHCl₃-MeOH mixture (200:1-1:1). Similar fractions were pooled and separated by column chromatography with Sephadex LH-20 gel using pure MeOH. Fractions were examined with thin layer chromatography (TLC) and pooled based upon the presence of xanthatin, based on published R_f values and TLC spot colors (Picman et al. 1980). The identity of the resulting compound was verified based on UV, MS, and ¹H-¹H ROESY NMR spectra.

Individual source populations were chemotyped via reverse phase high performance liquid chromatography (HPLC) with mass spectral and/or UV detection. Sesquiterpene lactones were extracted from bulk dry leaf material (collected from 5+ plants per population) in chloroform, dried down *in vacuo*, and dissolved in acetonitrile. Analyses were performed on either a Varian Prostar HPLC system (Agilent Technologies) with binary UV detection, or on an Agilent 1100 series HPLC system

(Agilent Technologies) with a UV detector coupled to a Bruker Esquire 6,000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Samples were analyzed using a Nucleodur Sphinx RP column (250 × 4.6 mm, 5 μm; Macherey-Nagel, Düren, Germany). Mobile phases were 0.2% formic acid (v:v) water and acetonitrile, with a gradient starting with 30% acetonitrile moving to 50% acetonitrile over 40 minutes. Under this protocol, the diastereomers 8-epi-xanthatin (*cis*) and xanthatin (*trans*) have characteristic retention times (20.4 and 21.1 minutes, respectively) and are identified by characteristic UV and MS spectra. The presence or absence of these peaks at absorbing at 280 nm was used to classify each source population as *cis*, *trans*, or mixed. Mixed populations (containing both *cis*- and *trans*-fused lactones) do exist, but were rare in our sampling (one of 26 collections, <4%) and were not used in the experiments described below. No populations were found that did not contain either 8-epi-xanthatin or xanthatin.

To verify that differences in retention time were in fact due to differences in stereochemistry, extracts from populations with *cis*- and *trans*-fused STL were spiked with pure xanthatin (*trans*), and peaks were observed. In samples with *trans*-fused lactones, the added xanthatin co-eluted with the xanthatin in the sample, resulting in a larger peak. In samples with *cis*-fused lactones, the added xanthatin formed a separate peak, as it did not co-elute with 8-epi-xanthatin. With one sample (TEN), numerous injections were made on an analytical HPLC, peaks were collected with an automated fraction collector, and then dried *in vacuo*. Samples were re-eluted in CDCl₃ and NMR experiments were performed on a Bruker Avance 400 MHz NMR spectrometer (Bruker Daltonics, Bremen, Germany). Based on differences in the ¹H-¹H ROESY spectra

between the samples, the two fractions were confirmed as 8-epi-xanthatin (peak 1) and xanthatin (peak 2).

Common Garden Sites. Research was conducted at two sites in southeastern Texas, the Katy Prairie Conservancy Research Station (hereafter KPC, N 29° 55' W 95° 55') and the University of Houston Coastal Center (hereafter HCC; N 29° 22' W 95° 2'). Both sites were historically dominated by coastal tallgrass prairie and have naturally occurring *Xanthium strumarium* populations nearby (<200m). In all experiments, fields were tilled in early April to provide disturbance.

Experiment 1 (2008). On 8 May 2008, we established a common garden experiment at KPC. Twenty five seedlings from each of eight *cis* populations and eight *trans* populations (totaling 400 seedlings) were planted in a completely randomized design. Plants were arranged in a grid spaced two meters apart and were watered every 2-3 days for one week following planting to increase transplanting success. Over the course of the growing season, leaf damage was estimated (methods below) for each plant three times (19 June, 17 July, 8 September). Under the terms of our research permit, we were required to harvest plants prior to bur drop, and thus were unable to measure plant fitness.

Experiment 2 (2009). In May 2009, we established common gardens at both KPC and HCC. In this year, our research permit at KPC was revised to allow bur formation, which allowed us to estimate fitness at both sites. At each site, twelve seedlings from each of 19 populations (eleven *cis* and eight *trans*) were planted in a completely randomized design (totaling 228 per site, 456 total). Plants were arranged in a grid with 1.5 m spacing. After planting, we watered for one week to increase

establishment success. Due to extreme drought conditions, plants at KPC were watered periodically through the growing season to increase survival. Twice during the growing season, surrounding vegetation was mowed and hand clipped to reduce aboveground competition. The 12 plants from each population were divided equally among the three damage manipulation treatments: reduced (insecticide), ambient (control), and elevated (mechanical damage). Damage was reduced by spraying with insecticide (Esfenvalerate), while other plants were sprayed with water as a control. Sprays were applied once per week from planting until mid-July, and once every two weeks afterwards. Plants in the elevated damage treatment were mechanically damaged monthly starting in July by clipping off half of each leaf with scissors. This level of damage (50% leaf removal) is within the upper range of damage naturally observed in the field (Ahern, unpublished data). Herbivore damage (see below) was surveyed three times for each plant (HCC: 17 June, 15-16 July, 10-12 August; KPC: 22 June, 19 July, and 18 August).

Trait Measurements. *Leaf Damage.* On each survey date, leaf damage by chewing insects was visually estimated on three (2008) or four (2009) haphazardly selected mature leaves. Damage was divided into two classes based on characteristic feeding patterns: holes (Orthoptera & Lepidoptera) and window-feeding (Lepidoptera & Coleoptera).

In Experiment 1 (2008), the percentage of total leaf area affected by each type of damage was estimated and assigned a score from 0 to 4, where 0 = no damage, 1 = 0.1%–5% damage, 2 = 5.1%–25% damage, 3 = 25.1%–50% damage, and 4 = 50.1%–100% damage. These scores were combined to generate damage scores (D), which represent total leaf damage per damage class.

$$D = \sum_{i=1}^4 \frac{n_i(C_i)}{N}$$

Here, i is the damage category (1-4), n_i is the number of leaves in the i th category, C_i is the midpoint of each damage category (i.e. $C_2 = 15.05$), and N is the total number of leaves scored per plant (nine). Final analysis was done on the sum of the damage scores across holes and window-feeding.

In Experiment 2 (2009), the same damage classes were examined, but damage was estimated to the nearest 5% in order to obtain a more precise estimate. In this case, D scores were generated by summing these raw estimates, and dividing by the total number of leaves surveyed ($n=12$). As before, analysis was done on the sum of the D scores for holes and window-feeding. Additionally, the percentage of leaf area removed by chewing insects (combined damage classes holes & window-feeding) was visually estimated for every leaf on each plant, providing a more accurate estimate of total percentage leaf area removed by chewing insects. Percentage leaf area was estimated as described above, with the exception of mechanically damaged leaves where mechanically removed tissue was included in this score.

Fitness. In Experiment 2 (2009), all burs from each plant were collected after plants had naturally senesced. Burs were sorted by hand to remove undeveloped, immature burs, and then counted. Mean bur mass varied dramatically across source populations (range: 95 mg - 566 mg) and bur mass was strongly positively correlated with seed mass ($r = 0.9438$, $P = <0.0001$, $n = 251$). Seed mass is commonly positively associated with germination rate, seed longevity, seedling competitive ability, and seedling survival in numerous species (Silvertown 1989, Sadras 2007); furthermore, in *X. strumarium*, seed size is known to influence germination rate, with larger seeds

germinating faster (Zimmerman and Weis 1983). Because of these considerations, we used the product of bur number and bur mass as our estimate of lifetime reproductive fitness. Results from alternate analyses using bur number as the fitness metric did not differ qualitatively from the results presented below (data not shown).

Statistical Analysis. All analyses were carried out in SAS (version 9.1; SAS Institute 2003). Response variables were \log_{10} transformed to achieve normality of residuals. In preliminary analyses of fitness patterns at HCC (see below), normality of the residuals could not be achieved without removing six outliers from the dataset. However, removal of these outliers did not qualitatively change the results of the analyses, and thus all data points were used in the final analysis. Due to differences in sample sizes (between years), and measured variables (within Experiment 2), the three site/year combinations were analyzed separately.

To investigate whether there was heritable variation in herbivore resistance, we performed analysis of variance (ANOVA) in Proc GLM. In this model, combined damage was the response variable, source population was treated as a fixed factor, and individual plants were the units of replication. For the two gardens in 2009 we excluded pesticide treated and mechanically damaged plants, solely investigating plants with natural levels of leaf damage. Tukey's post-hoc pair wise comparisons were used to examine which populations differed significantly from one another. We calculated broad-sense heritability (H_B^2) for resistance to chewing insects in all gardens. Broad sense heritability, the degree of genetic determination, estimates the overall genotypic contribution to the phenotype (Falconer and Mackay 1996). It is calculated by dividing V_G , the total genetic variation (a combined estimate of additive and non-additive genetic

variance) by V_P , the total phenotypic variance. We calculated V_G and V_P from the ANOVA results as described in (Falconer and Mackay 1996). Broad-sense heritability likely overestimates the evolutionary potential of a group because it includes both the additive and non-additive genetic variance components, and thus serves as an upper limit to the heritability of a trait (Falconer and Mackay 1996). Additionally, because we used seed collected from natural populations in our experimental gardens, we are making the assumptions that a) populations are genetically uniform and inbred, and b) maternal effects are limited or non-existent. The first assumption is reasonable, given that inbreeding is common in *X. strumarium*, often resulting in little or no allelic polymorphism within a population (Moran and Marshall 1978, Dinelli et al. 2003). However, the validity of the second assumption is not clear. Maternal effects can inflate an estimate of H_B^2 by adding a non-genetic component of the overall phenotypic variance into the genetic component (Falconer and Mackay 1996). Because of these considerations, our estimates of H_B^2 should be interpreted as upper bounds.

To assess whether stereochemistry influenced herbivore resistance, we conducted nested ANOVA in Proc Mixed. Combined damage was the response variable, stereochemistry was treated as a fixed effect, and source population was nested within stereochemistry as a random effect.

To investigate whether leaf damage reduced plant fitness, we conducted separate ANCOVA in Proc Mixed (one for each site in 2009). Plant fitness (seed number x seed mass) was the response variable, and percentage leaf damage, stereochemistry, and source population were predictors. As above, stereochemistry was treated as a fixed effect, and source population was nested within stereochemistry as a random effect. We

parameterized this model to fit separate intercepts and slopes for the different plant chemotypes. Significant negative slopes indicated that damage reduced fitness. We then used adjusted means contrasts to conduct pair wise tests to examine whether slopes differed between chemotypes, interpreting significant differences as evidence that chemotypes differed in their fitness response to herbivore damage. Importantly, because we imposed damage, differences in slope can inform us about the relative tolerances to leaf damage of the chemotypes, with a steeper negative slope indicating a less tolerant chemotype (Tiffin 2000). Differences in the intercept between chemotypes would indicate differential fitness in the absence of damage.

To assess the relationship between stereochemistry and plant fitness in the two 2009 gardens, we conducted nested ANOVA in Proc Mixed. Plant fitness was the response variable, stereochemistry was treated as a fixed effect, and source population was nested within stereochemistry as a random effect. To examine fitness differences between chemotypes in response to ambient levels of damage, we first excluded pesticide treated and mechanically damaged plants from the analysis. Then, to examine differences between chemotypes under reduced levels of damage (i.e., in the absence of the putative selective agents), we excluded control and mechanically damaged plants from the analysis.

2.3 Results

Is there heritable variation in herbivore resistance in *Xanthium strumarium*?

Source populations differed dramatically in herbivore resistance. In 2008 at KPC, mean leaf damage ranged from 1.8 to 17.4 % across populations, almost a ten fold difference ($F_{15,360} = 20.25$, $P < 0.0001$, Figure 2.2a). Similar patterns were apparent in 2009, with a

5.4 fold difference among populations at KPC ($F_{17,49} = 2.06$, $P = 0.0377$, Figure 2.2b) and a 12 fold difference at HCC ($F_{17,67} = 1.63$, $P = 0.0923$, Figure 2.2c). Note that the marginal significance of the HCC result is likely due to the combination of smaller sample sizes and lower damage levels observed during 2009. Broad sense heritability (H_B^2) estimates were high in all experimental gardens: 0.955, 0.673, and 0.619 for KPC 2008, KPC 2009, and HCC 2009 respectively.

Is stereochemistry associated with herbivore resistance? Plants producing *cis*-fused lactones consistently experienced higher levels of herbivory in the field. This pattern held across both years at KPC (2008, $F_{1,14} = 27.54$, $P = 0.0001$; 2009, $F_{1,16} = 25.8$, $P = 0.0001$, Figures 2.3a & 2.3b), and at HCC in 2009 ($F_{1,16} = 8.85$, $P = 0.0089$; Figure 2.3c).

Does herbivore damage reduce fitness? Manipulative experiments in 2009 revealed that damage reduced plant fitness, although the strength of the relationship differed across sites and chemotypes (Table 2.2 and Figure 2.4). At KPC, there was a trend that *cis* plants had a significantly negative slope ($F_{1,16} = 3.31$, $P = 0.0879$), while *trans* plants had a non-significant slope. However, at HCC both *cis* and *trans* plants had highly significant negative slopes (*cis*, $F_{1,17} = 28.41$, $P < 0.0001$; *trans*, $F_{1,17} = 19.36$, $P = 0.0004$). Within each site, the slopes of *cis* and *trans* plants did not significantly differ from each other, indicating they do not differ in tolerance to herbivory (Table 2.2).

As expected from the above results linking stereochemistry to resistance, stereochemistry was also linked to plant fitness. Under ambient levels of damage (the control treatment), plants with *trans*-fused STL had higher fitness than plants with *cis*-fused lactones (KPC, $F_{1,17} = 4.52$, $P = 0.0486$; HCC, $F_{1,17} = 5.31$, $P = 0.0341$). However,

under reduced levels of herbivory chemotypes did not differ in fitness (KPC, $F_{1,17} = 0.30$, $P = 0.5936$; HCC, $F_{1,17} = 3.00$, $P = 0.1006$).

2.4 Discussion

Through the use of differentiated populations of *Xanthium strumarium* grown in common garden environments, we found evidence of extensive population-level variation in resistance to chewing insects. Calculations of broad-sense heritability indicated that there is substantial heritable variation for herbivore resistance in this species. Baseline chemotyping separated source populations into two chemotypes based on the lactone ring junction: plants producing *cis*- or *trans*-fused sesquiterpene lactones. This simple chemical marker was strongly and consistently correlated with foliar herbivory in the field, with plants producing *cis*-fused lactones receiving more damage than plants producing *trans*-fused lactones. In addition, experiments manipulating foliar herbivory above and below ambient levels found that for both chemotypes leaf damage reduced plant fitness. Finally, lactone ring junction stereochemistry influenced plant fitness under ambient levels of damage, with plants producing *trans*-fused lactones having significantly higher fitness than plants with *cis*-fused lactones. However, under experimentally reduced levels of damage this pattern disappeared, suggesting that there are not substantial differences in the costs of *cis*-fused vs. *trans*-fused lactones in the absence of herbivores.

Potential mechanisms underlying differential bioactivity between *cis*- and *trans*-fused sesquiterpene lactones. Sesquiterpene lactones are known to have bioactivity against a broad array of organisms due to their ability to alkylate biological macromolecules. The α,β -unsaturated carbonyl groups commonly found associated with

the lactone moiety of STL are believed to be predominant source of bioactivity of STL (Schmidt 1999). These groups readily react with nucleophilic functional groups in biological macromolecules such as the sulfhydryl group of cysteine residues via a Michael addition reaction (Schmidt 1999). This reaction often leads to the deactivation of enzymes and bioactive proteins, although other targets and mechanisms have also been proposed (Schmidt 1999). While the presence of a α,β -unsaturated carbonyl group appears to be the essential component conferring bioactivity, the extensive variability in bioactivities between tested STL indicates that other features of the compounds also contribute to bioactivity (Schmidt 2006). Investigations of structure-activity relationships of STL have found that several other factors influence activity, including oxygenation state, molecular size, polarity/hydrophilicity, and skeletal type (Schmidt 2006).

In comparison to these other factors, we know relatively little about how stereochemical variation influences biological activity in STL. However, laboratory studies have demonstrated that stereochemical variation can influence bioactivity *in vitro* (Beekman et al. 1997, Takeda et al. 2011, Ahern Chapter 1). Observed differences in bioactivity between STL stereoisomers could potentially arise via at least two different (but not mutually exclusive) mechanisms: ring strain-based activity and target-based activity. Ring strain is a destabilizing force that acts on cyclic molecules in unfavorable angular configurations. In regard to stereochemical variation in STLs, *trans*-fused lactones should have more ring strain because of the angles by which the lactone ring is bonded to the remaining portion of the molecule. As a result, *trans*-fused lactones should have higher energy and should thus act as stronger electrophiles with greater bioactivity. This potential mechanism is in accordance with the data presented in this paper and

elsewhere, indicating higher bioactivity of *trans*-fused lactones (Takeda et al. 2011, Ahern Chapter 1).

Alternatively, differences in bioactivity between chemotypes could be due to differential target-based activity. The three-dimensional shape and flexibility of a molecule dictates whether and how efficiently it can interact with a target site. This interaction will be particularly important if access to targets is restricted, e.g. if targets are located in hydrophobic pockets of enzymes (Schmidt 1999). Beekman *et al.* found compounds with *cis*-fused lactones were more cytotoxic to a mouse cancer cell line than *trans*-fused lactones (Beekman et al. 1997). The authors hypothesized the observed differences were due to the stereochemistry of the lactone ring junction because, in the STL under study, compounds with *trans*-fused lactones were rigid, while those with *cis*-fused lactones were flexible and thus potentially able to interact with more restricted targets (Beekman et al. 1997). Importantly, if STL chemotypes have differential target-based activity, generalities may not be available as to whether *cis*- or *trans*- forms have greater bioactivity: the bioactivity of a STL will not only be due to its three-dimensional structure, but also to that of its target site. Plant defensive chemicals potentially interact with a number of different molecular targets when consumed by herbivores (e.g. chemoreceptors, peritrophic membranes, detoxification enzymes (Barbehenn 2001, Chapman 2003, Li et al. 2007). Thus, we may not be able to make general predictions about the bioactivities of stereoisomers.

Influence of stereochemical vs. other chemical variation. We have demonstrated strong correlations between STL lactone junction stereochemistry and patterns of chewing damage in the field. These results match those from laboratory

feeding assays, indicating *trans*-fused STL are more deterrent to chewing insects than *cis*-fused STL (Ahern, Chapter 1). However, the possibility remains that other traits correlated with STL stereochemistry, e.g. differences in the relative or absolute abundances of different STL compounds, could also contribute to the patterns seen here. For example, plants producing *trans*-fused STL may benefit not only from a more bioactive stereoisomer, but could conceivably also produce more overall STL, or produce higher quantities of a more deterrent or toxic form than those produced by plants with *cis*-fused STL. STL profiles in *X. strumarium* are extremely variable: over 39 unique sesquiterpene lactones have been identified in *Xanthium* to date (Zhang et al. 2001), and up to twelve unique sesquiterpene lactones have been documented from a single population (e.g. Ahmed et al. 1990). Further, different source populations often differ both quantitatively and qualitatively in their STL composition (McMillan et al. 1975a, McMillan et al. 1975b). This variation in STL profiles would likely help explain the differences in herbivore resistance between individual source populations (Figure 2.2) that cannot be explained by stereochemical variation alone. Quantifying such non-stereochemical variation, in conjunction with stereochemical variation, and examining how they might jointly explain variation in herbivore resistance is an important area of future work. Nonetheless, the results of our previous laboratory feeding trials have clearly demonstrated that STL lactone junction stereochemical variation influences herbivore feeding deterrence. The consistency of these previous findings with data from field experiments spanning multiple sites and years (Figure 2.3) suggests that STL lactone junction stereochemical variation makes a major contribution to variation in herbivore resistance.

Natural selection on sesquiterpene lactones mediated via insect herbivores.

We have demonstrated herbivore-mediated natural selection acting on variation in STL chemistry. The criteria necessary to demonstrate selection by an herbivore on plant traits are 1) intraspecific genetic variation in damage; 2) intraspecific genetic variation in a plant trait that is correlated with damage, and 3) correlation between the trait (and damage) and plant fitness (Marquis 1992). For the first criterion, we found significant variation in resistance to insect herbivores between source populations. Broad sense heritability estimates based on this data suggest that this variation is highly heritable, as calculations of H_B^2 ranged between 0.619-0.955. We found strong associations between STL stereochemistry and resistance, satisfying the second criterion. Finally, both STL stereochemistry and leaf damage were correlated with plant fitness, satisfying the third criterion. Together, these three lines of evidence indicate that there is natural selection on STL mediated via herbivores. Although it is commonly assumed that herbivores drive the evolution of many classes of plant secondary metabolites, herbivore-mediated selection on defensive chemicals has rarely been demonstrated (but see: Berenbaum et al. 1986, Lankau 2007), and to the best of our knowledge, this the first documented example of natural selection on stereochemical variation in defensive chemicals.

Maintenance of *cis* and *trans*-stereoisomers in *Xanthium strumarium*. An important question remaining is, how is the less bioactive, *cis*-fused STL form being maintained within *X. strumarium*? Despite differences between chemotypes in herbivore resistance, they did not differ in tolerance (i.e., the slopes of the regressions of fitness on damage). Thus, a tradeoff between resistance and tolerance (as has been demonstrated in other systems, e.g. (Leimu and Koricheva 2006)) does not provide a likely explanation

for the maintenance of the *cis*-chemotype in *X. strumarium*. Other forces must be at work. Although the patterns observed through the course of these experiments showed that *trans*-fused STL confer more herbivore resistance, there may be places or times where *cis*-fused STL confer equal or higher resistance. We are currently completing field experiments in multiple environments to uncover locations where each chemotype has the highest fitness, and thus to evaluate whether spatial variation in natural selection maintains stereochemical variation in STL. Furthermore, comparative methods investigating the phylogenetic patterns of STL stereochemistry across lineages of plants could be informative about the factors driving the evolution of stereochemical variation in STL. Multiple techniques and approaches will likely be necessary to elucidate the factors driving small- and large-scale patterns of variation in this class of compounds.

Stereochemistry in plant defense. Numerous classes of plant secondary metabolites contain extensive stereochemical variation, including terpenoids, alkaloids, flavonoids, coumarins, and glucosinolates (based on examination of data in Buckingham 2011). Despite this ubiquity, research investigating the importance of stereochemistry in mediation of plant-herbivore interactions has thus far been confined to a limited number of case studies. Current evidence suggests that stereochemistry can have large effects on both herbivore preference and performance. For example, mints (*Mentha spp.*) contain the enantiomeric monoterpenes (*R*)-(+)-pulegone and (*S*)-(-)-pulegone, but only the former compound showed significant feeding deterrence against the aphid *Myzus persicae* in laboratory bioassays (Dancewicz et al. 2008). Demonstrative studies such as this, along with the evidence accumulated from the fields of pesticide and pheromone science, indicate that we should pay closer attention to the stereochemistry of plant

secondary metabolites, as this often overlooked factor may play an important role in biological activity.

Stereochemistry may be particularly important in compounds formed via complex, multi-step biosynthetic pathways, because stereochemical differentiation can occur early in the biochemical pathway, affecting all downstream compounds. For example, polymeric condensed tannins are composed of chains of monomers, e.g. catechin, that show stereochemical variation. Different ratios of catechin:epicatechin and thus *cis*- vs. *trans*-fused bonds in condensed tannins influenced snowshoe hare preference for different diets (Clausen et al. 1990). This finding supports recent calls for more in-depth analyses of tannin profiles, as assays of ‘total tannin content’ overlook the inherent complexity of the constituent chemicals (Salminen and Karonen 2011). A similar case can be made for highly modified terpenoids like the sesquiterpene lactones. The stereospecific hydroxylation and subsequent lactonization of sesquiterpenes appears to be one of the early steps in sesquiterpene lactone biosynthesis (de Kraker et al. 2002, Ikezawa et al. 2011). It is proposed that once the few initial lactones are formed (by different orthologs of the p450 enzyme *LsCOS*), subsequent downstream modifications lead to the vast diversity of sesquiterpene lactones (Fischer et al. 1979, Ikezawa et al. 2011). If a given plant only expresses a single ortholog of this enzyme (as appears to be the case in *X. strumarium*), the resulting products all have the same lactone junction stereochemistry (either *cis* or *trans*). Thus, the stereochemistry of a single bond can cascade to characterize a plant’s defensive chemical profile, influencing herbivore resistance and plant fitness.

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2.7 Tables

Table 2.1: Locality information for *X. strumarium* accessions including identification codes, location, latitude and longitude of seed sources. Information is also provided on the stereochemistry of the lactone ring junction (*cis* vs. *trans*), and utilization of the accessions in the two experiments.

ID	Site of Seed Collection	State	Country	Latitude	Longitude	Experiment	
						1	2
<i>Cis</i>							
ANF	Angelina NF	Texas	USA	31°02'34" N	94°17'33" W		x
CYC	Cypress Creek	Texas	USA	29°57'57" N	95°36'25" W	x	x
GUA	Guangzhou	Guangdong	China	23°11'11" N	113°21'38" E	x	x
JEJ	Jessie Jones Katy Prairie	Texas	USA	30°01'52" N	95°18'9" W	x	x
KPC	Conservancy	Texas	USA	29°56'06" N	95°55'48" W	x	x
LAC	Lake Creek	Texas	USA	30°16'48" N	95°42'19" W	x	x
NC	Inglis Farm	North Carolina	USA	36°04'13" N	76°26'49" W		x
SJC	San Jacinto	Texas	USA	30°18'55" N	95°30'37" W	x	x
TEN	Tennessee	Tennessee	USA	36°23'50" N	83°27'28" W	x	x
WUH	Wuhan	Hubei	China	30°32'37" N	114°24'49" E	x	x
<i>Trans</i>							
AUS	Austin	Texas	USA	30°11'51" N	97°37'51" W	x	x
BRR	Brazos River	Texas	USA	29°34'3" N	95°48'35" W	x	x
FTS	Ft Stockton	Texas	USA	30°53'33" N	102°51'47" W	x	x
GOL	Goliad Houston	Texas	USA	28°42'4" N	97°23'42" W	x	x
HCC	Coastal Center	Texas	USA	29°23'27" N	95°02'34" W	x	x
JAC	Cozy Corner Recurrent	Texas	USA	29°51'18" N	96°50'34" W	x	x
RAR	Annuus	Texas	USA	32°31'26" N	96°24'10" W	x	x
WRR	Warren Ranch	Texas	USA	29°58'42" N	95°51'01" W	x	x

Table 2.2: Regressions of fitness on damage at two sites, HCC and KPC. In the main model, significant p-values indicate that a chemotype (either *cis* or *trans* plants) had a significantly negative slope, indicating that leaf damage reduced plant fitness. Contrast statements tested whether chemotypes differed in their fitness response to foliar herbivory.

KPC					
	Estimate	SE	DF	t value	P
<i>Cis</i>	-0.00747	0.004111	16	-1.82	0.0879
<i>Trans</i>	-0.00458	0.004031	16	-1.14	0.2724
	Contrast	Num	Denom	F	P
	<i>Cis vs. Trans</i>	1	16	0.25	0.6224

HCC					
	Estimate	SE	DF	t value	P
<i>Cis</i>	-0.05284	0.08032	17	-5.33	<.0001
<i>Trans</i>	-0.04793	0.01088	17	-4.4	0.0004
	Contrast	Num	Denom	F	P
	<i>Cis vs. Trans</i>	1	17	0.11	0.743

2.8 Figures

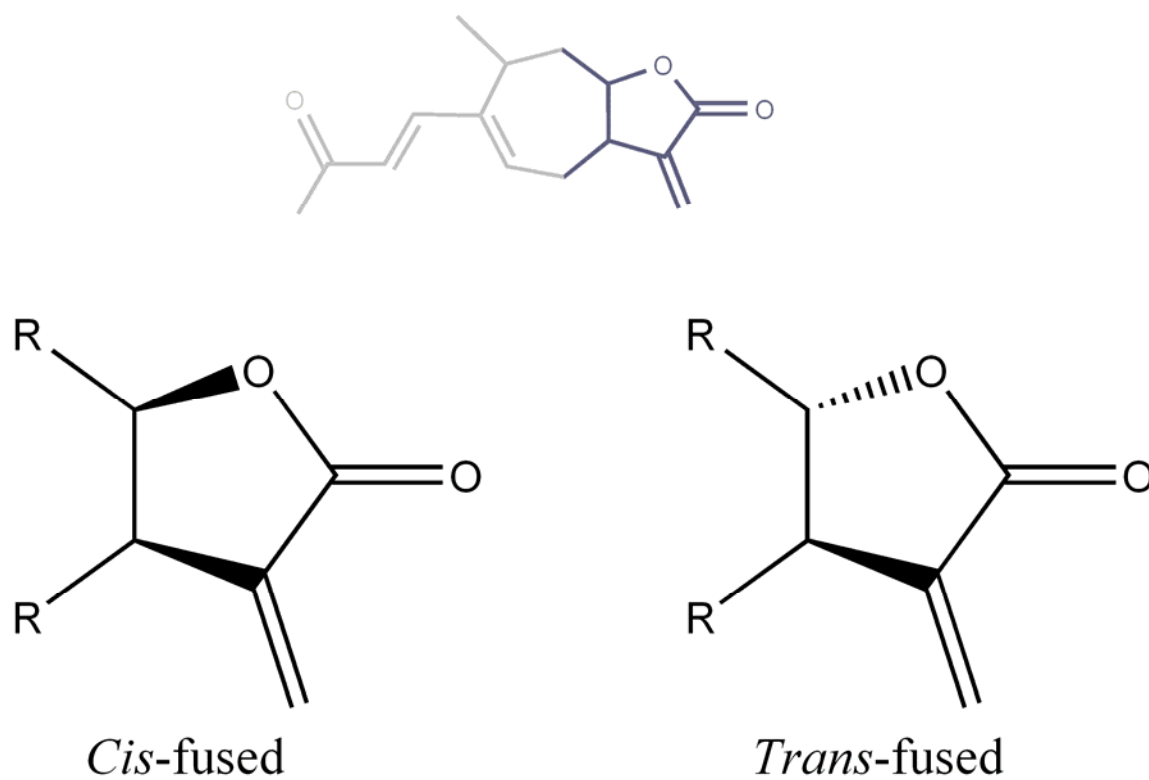


Figure 2.1 In *Xanthium strumarium*, plants differ in the stereochemistry of the lactone ring junction. Pictured on the left is the lactone moiety of a *cis*-fused lactone ring, on the right is that of a *trans*-fused lactone ring.

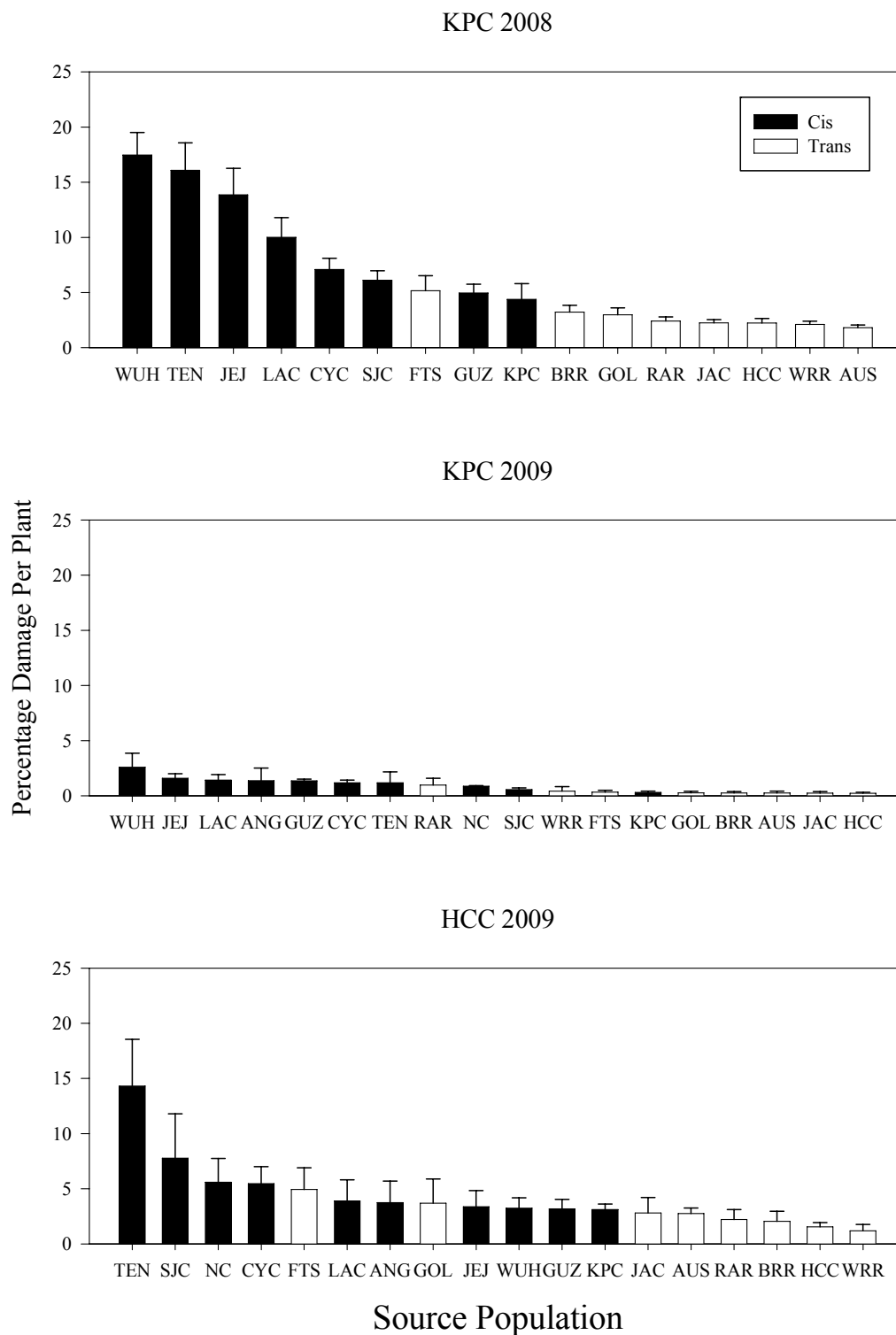


Figure 2.2. Populations differed dramatically in herbivore resistance in 2008 (a), and similar patterns were apparent in 2009 (b-c). The three panels depict results for common gardens at Katy Prairie (2008, 2009) and the Houston Coastal Center (2009). Data are presented as least squares means (\pm) standard errors.

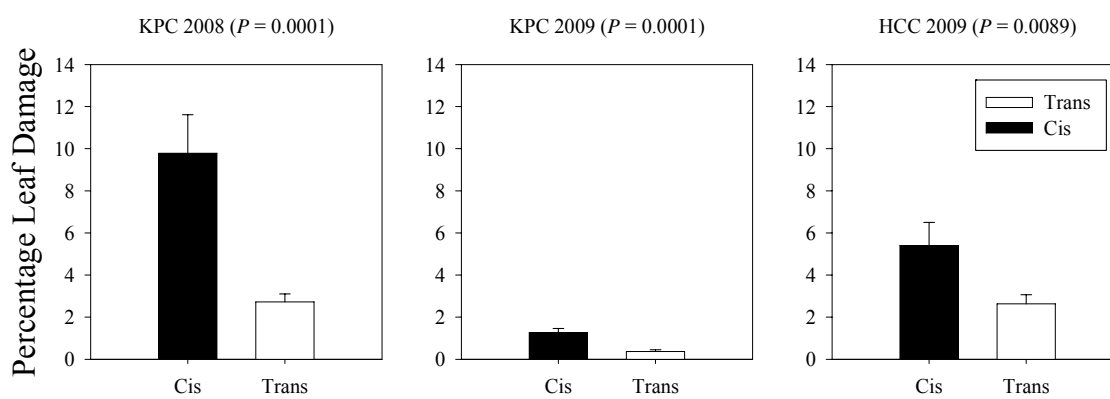


Figure 2.3. Plants producing *cis*-fused lactones consistently experienced higher levels of herbivory in the field. Data are presented as least squares means (\pm) standard errors.

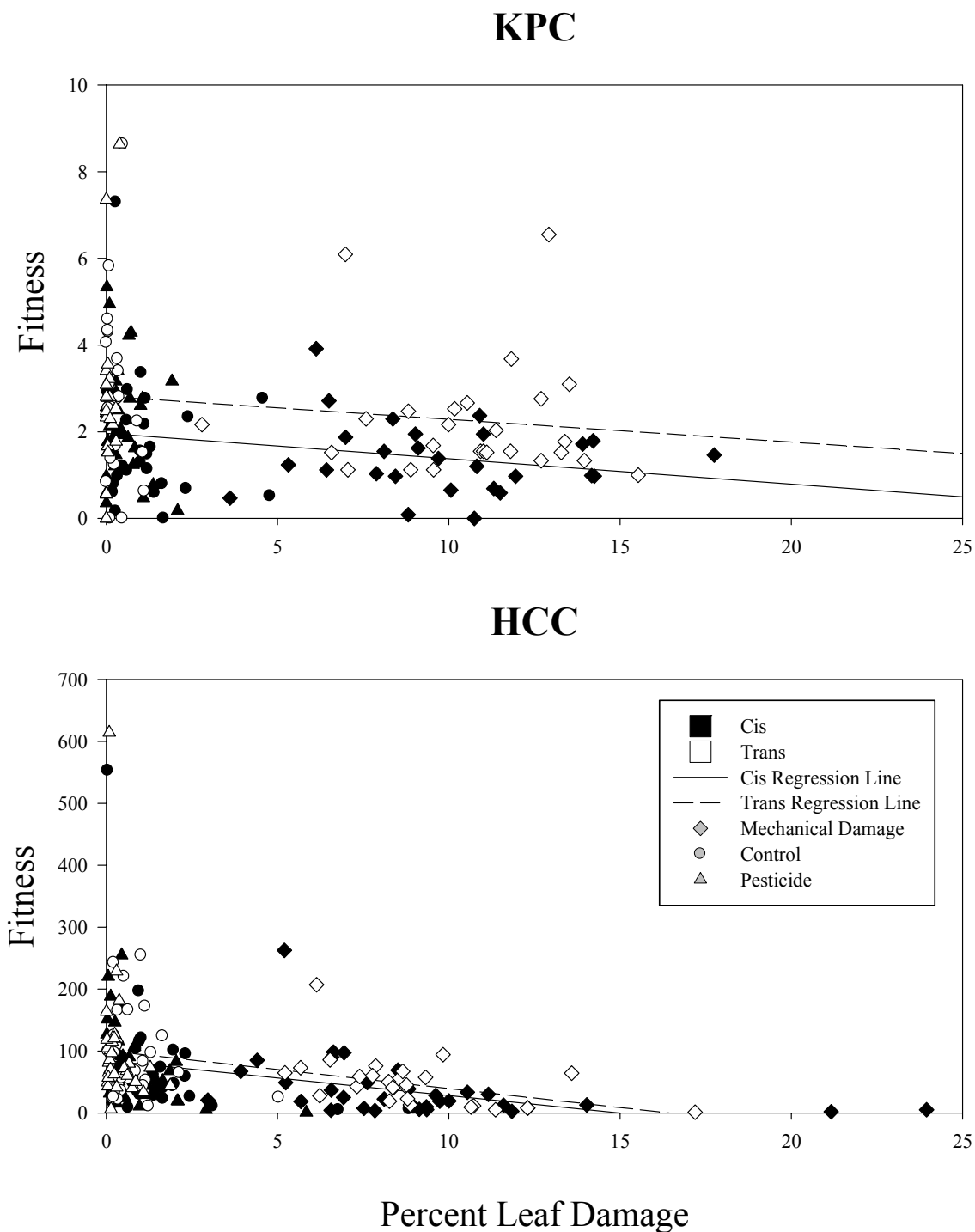


Figure 2.4. Relationship between leaf damage and fitness (bur number x bur mass) for *X. strumarium* in two sites (2009). Black symbols indicate *cis* plants, white symbols indicate *trans* plants. Diamonds indicate plants that received mechanical damage via scissors, Circles indicate control plants that received ambient damage levels. Triangles indicate plants were sprayed with pesticides.

Chapter 3

Evidence That Herbivore-Mediated Spatially Variable Selection Contributes to the Maintenance of a Defensive Chemical Trait Polymorphism

The factors that maintain defensive chemical variation within and between plant species have intrigued ecologists for decades. However, surprisingly few experimental studies have examined the selective mechanisms responsible for the maintenance of polymorphisms in nature. Here, we report on a chemical trait polymorphism in a widespread annual weed, *Xanthium strumarium*, in which plants produce sesquiterpene lactones with either a *cis*- or *trans*-fused lactone ring. Using five common gardens spread throughout eastern Texas, we tested whether spatial variation in the intensity of folivore damage imposes variable selection on this polymorphic trait. We detected significant GxE interactions on plant fitness, such that each morph had equal or higher fitness in different environments. Furthermore, this effect was related to the intensity of folivore damage at a site: plants producing *trans*-fused sesquiterpene lactones had higher relative fitness in sites with high levels of folivore damage, whereas plants with *cis*-fused sesquiterpene lactones had equal or higher relative fitness in sites with low damage. These results highlight the importance of herbivore-mediated variable selection for the maintenance of defensive chemical variation.

3.1 Introduction

Plants have evolved a vast diversity of defensive chemicals (secondary metabolites with a defensive role) that serve to prevent or reduce damage from natural enemies. Explaining the diversity and function of secondary metabolites has been a goal of phytochemists and chemical ecologists for more than half a century (Dethier 1954, Fraenkel 1959, Ehrlich and Raven 1964, Jones et al. 1991, Berenbaum and Zangerl 1996, Berenbaum and Zangerl 2008). Much of this work posits that herbivores and other natural enemies impose natural selection on plant defensive chemicals, driving diversification between clades. Since most natural selective mechanisms should remove variation from a population (Falconer and Mackay 1996), one might expect secondary metabolites to exhibit little natural variation. Instead, inter- and intraspecific variation in secondary metabolites is nearly ubiquitous (Mabry 1973, Hunter et al. 1996). Numerous hypotheses have been proposed to explain variation in plant defenses; these mainly center on costs of defense (e.g. genetic, ecological, structural; reviewed in Stamp 2003, Strauss et al. 2002) as key mechanisms to maintain variation. However, despite these theoretical frameworks, there is limited empirical evidence demonstrating the ecological and evolutionary factors that serve to maintain variation in plant defensive chemistry.

Discrete polymorphisms in plant defensive chemicals provide tractable systems for examining the ecological and evolutionary basis behind the maintenance of variation in plant defenses because the simplified nature of polymorphic traits facilitates the study of natural selection in natural systems (e.g. Epling and Dobzhansky 1942, Sheppard 1951). Discrete polymorphic traits are often controlled by a single or a few loci of large

effects, which allows the application of a classic Mendelian genetic framework (Alonso-Blanco et al. 2005).

Although natural selection and genetic drift typically remove variation from populations, there are three selective mechanisms that can maintain genetic polymorphisms in nature: overdominance (O'Donald 1967, Hedrick 2012), negative frequency dependent selection (Ayala and Campbell 1974), and variable selection (Hedrick et al. 1976, Hedrick 1986, 2006), collectively referred to as balancing selection. To implicate overdominance, one must demonstrate that individuals heterozygous at the locus underlying the trait of interest have higher fitness than either homozygous morph. For negative frequency dependent selection, one must demonstrate that each morph has higher fitness when at a low frequency in the population. To implicate variable selection, one must demonstrate that each morph has equal or higher fitness in different places or times. Although a myriad of polymorphic traits have been proposed to be selectively maintained in nature, in an overwhelming majority of cases, the appropriate experiments to detect the signature of one or more of these selective mechanisms have not yet been conducted.

Defensive chemical trait polymorphisms have been identified and studied extensively through the years. Table 3.1 presents a list of examples that we believe constitute the best demonstrations of defensive chemical trait polymorphisms maintained by natural selection. These examples include the glucosinolate polymorphisms in *Barbarea vulgaris* and *Boechnera stricta*; monoterpene polymorphism in *Thymus vulagris*, the cyanogenesis polymorphisms in *Trifolium repens* and *Lotus corniculatus*, and the trichome type polymorphism in *Datura wrightii* (Table 3.1 for references). All of these

systems have discrete qualitative heritable variation in chemical traits that are correlated with damage from natural enemies. This indicates that these polymorphic traits a) serve a defensive function and b) can respond to natural selection. The persistence of these polymorphisms in natural populations suggests they are selectively maintained.

However, despite the extensive research effort invested, there is surprisingly little experimental evidence demonstrating one or more of the three mechanisms for maintaining defensive chemical trait polymorphism. Many researchers have simply not yet conducted the appropriate tests to detect signatures of balancing selection that could maintain a defensive chemical trait polymorphism. First, in many cases, reproductive fitness has not been quantified for plants growing in field settings (e.g. Jones 1966, van Leur 2008, Schranz et al. 2009, Manzaneda et al. 2010). Second, amongst those studies that have examined plant fitness, many have not followed fitness through the entire lifespan of the plants (e.g. Daday 1965, Ennos 1981, Dirzo and Harper 1982, Linhart et al. 1999, Elle and Hare 2000). This is particularly problematic with perennial iteroparous plants, where lifetime reproductive fitness is difficult to quantify *and* fitness estimates based on single year of growth can lead to inconclusive or spurious results. For example, a three year experiment revealed differences in the finite rate of increase (λ) between trichome type morphs of the perennial forb *Datura wrightii* (Hare et al. 2003) that were not apparent in a single season of viable seed production (Elle et al. 1999). Amongst those studies that have adequately quantified plant fitness, most have failed to find evidence for mechanisms leading to the stable maintenance of the polymorphic trait (negative frequency dependent selection, overdominance, or variable selection, Table 3.1).

We illustrate these issues with the case study on the cyanogenesis polymorphism in *Trifolium repens*. This polymorphism has been examined for nearly a century (Armstrong et al. 1913), and is often considered a classic example of a selectively maintained polymorphism (Hughes et al. 1988). Cyanogenic plants are believed to have more resistance to herbivores than acyanogenic plants, at a cost of resource allocation and reduced freezing tolerance (Hughes et al. 1988). Recent molecular evidence indicates clines have formed rapidly in novel environments, with signatures of adaptive evolution (Kooyers and Olsen 2012). Thus, selection via herbivores and climate has been hypothesized to maintain variation in cyanogenesis, both locally and regionally. However, despite decades of ecological experiments (reviewed in Hughes 1991), evidence implicating the ecological selective forces responsible for the maintenance of the polymorphism across a range of scales has not yielded a conclusive explanation. Individual studies have documented superior fitness components of cyanogenic morphs in some environments (e.g. Daday 1965, Ennos 1981, Saucy et al. 1999) and acyanogenic morphs in others (e.g. Daday 1965, Dirzo and Harper 1982), yet, to our knowledge, only a single study has shown superior fitness components of each morph in different natural environments (proportion of flowering plants, but not seed number, Daday 1965). As with the *Thymus* system, fitness differences in each environment were associated with temperature differences rather than herbivores. Although it may be tempting to combine evidence from multiple studies to assess the net selective factors associated with the maintenance of this polymorphic trait, genotypic differences between source populations could lead to spurious results. Genotypic variation between different accessions of *Trifolium repens* may vary in other traits that could account for observed fitness

differences in experimental settings. For example, Olsen and Ungerer (2008) found that acyanogenic plants had superior freezing tolerance than cyanogenic plants when examining responses of diverse accessions from around the world, but did not detect any differences in freezing tolerance when examining responses of plants from polymorphic populations. Thus, there is still a need to explicitly demonstrate evidence showing the selective basis behind the maintenance of a defensive chemical trait polymorphism.

Here, we set out to examine factors associated with the maintenance of a stereochemical trait polymorphism in *Xanthium strumarium*, a weedy annual plant with a global distribution. This species is polymorphic with respect to the stereochemistry of the lactone ring junction of a prominent defensive compound class – the sesquiterpene lactones. An individual plant will typically produce only *cis*-fused or *trans*-fused lactones across its entire suite of compounds (McMillan 1974, McMillan et al. 1975b). Experimental crossing studies indicate lactone junction stereochemistry is a co-dominant trait with simple Mendelian inheritance (Chavez 1973, McMillan et al. 1975a). Current understanding of sesquiterpene lactone biosynthesis suggests that *cis*- and *trans*-fused lactones are formed by different alleles of a single cytochrome p450 gene (Ikezawa et al. 2011, D. K. Ro, pers. comm.), indicating this polymorphism is caused by a single gene of large effect. Our previous work in this system has demonstrated that this stereochemical trait is a) heritable, b) correlated with both folivore damage and plant fitness in the field (Ahern, Chapter 2), and c) correlated with grasshopper feeding preference in controlled pure chemical feeding assays (Ahern, Chapter 1). Our previous evidence indicates that this trait is under phenotypic selection via herbivores in the field, and subject to evolutionary change (following criteria in Marquis 1991, 1992). Here, we examined

whether spatially variable selection could maintain the stereochemical trait polymorphism. We focused on this mechanism for two reasons. First, both forms are found throughout eastern Texas, yet typically occur as isolated, monomorphic populations (Chavez 1973, McMillan et al. 1975a) (mixed populations are rarely observed), suggesting that overdominance and negative frequency dependent selection are unlikely mechanisms. Second, in selfing species such as *X. strumarium*, temporally variable selection can maintain polymorphisms only under a highly restrictive set of conditions (Hedrick 1998), whereas under spatially variable selection, the conditions that maintain polymorphisms in selfing species are estimated to be approximately the same as for fully randomly mating populations (Hedrick 1998).

Thus, we set out to examine whether spatially variable selection imposed by folivorous herbivores is responsible for the maintenance of the stereochemical trait polymorphism in *Xanthium strumarium*. We specifically addressed: **1) Does stereochemistry affect folivore damage, and does this effect vary spatially?** **2) Does folivore damage and/or stereochemistry affect plant survival and reproduction?** **3) Is there spatial variation in the identity of the most fit morph, such that each stereochemical morph performs best in some sites?** Finally, **4) Does the intensity of folivore attack predict the identity of the most fit morph across sites?** To address these questions, we utilized a common garden approach with replicated source populations and established five gardens across southeastern Texas in 2010. In conjunction with our previous work (Ahern, Chapters 1 & 2), this study is the first, to our knowledge, to implicate herbivore-mediated spatially variable selection in the maintenance of a plant defensive chemical trait polymorphism.

3.2 Methods

Study System. *Xanthium strumarium*, the common cocklebur, is a cosmopolitan annual weed that inhabits ruderal and riparian habitats (Weaver and Lechowicz 1983). It is monoecious with spatially separated male and female flowers and, though wind pollinated, it is primarily an inbreeder because male flowers develop above female flowers, raining pollen directly onto them (Löve and Dansereau 1959, Weaver and Lechowicz 1983). This breeding system, along with periodic outcrossing, results in differentiated monomorphic populations, with much of the phenotypic variation found among, rather than within, populations. Phenotypic variation among populations is so extensive that over 50 species epithets were previously recognized in different floras in North America (Löve and Dansereau 1959). However, most current treatments recognize only a single species, e.g. Flora of North America Editorial Committee (1993+). Female flowers produce indehiscent two-seeded fruits (burs) that are dispersed by gravity, water, or animals via hooked spines on the surface of the bur (Weaver and Lechowicz 1983).

Xanthium strumarium is polymorphic in regards to variation in the stereochemistry of its prominent defensive chemicals, sesquiterpene lactones. The lactone ring junction of sesquiterpene lactones can be either *cis*- or *trans*-fused (Figure 2.1). In the species as a whole, both *cis*- and *trans*-fused lactones are found, but most natural populations are monomorphic (all of the plants produce either all *cis*-fused or all *trans*-fused STL, McMillan 1974, McMillan et al. 1975b). This trait is believed to be codominant with simple Mendelian inheritance (McMillan et al. 1975a), caused by allelic variation in the enzyme responsible forming the lactone ring junction (D. K. Ro, pers. comm.). In other species, it has been shown that this enzyme, a cytochrome p450

hydroxylase, is responsible for stereospecific ring formation (de Kraker et al. 2002, Ikezawa et al. 2011). This reaction occurs early in the biosynthesis of sesquiterpene lactones which subsequently undergo substantial modification, leading to numerous end products (Fischer et al., 1979).

Sesquiterpene lactones (STL) are believed to be defensive compounds, exhibiting biological activity against a broad array of organisms including against bacteria, fungi, invertebrates, and vertebrates (Picman, 1986). This bioactivity is most commonly linked α,β -unsaturated carbonyl groups, often found on the lactone ring (Schmidt, 1999). These chemical warheads bind to biological macromolecules via a Michael-type addition reaction, and often result in the deactivation of enzymes (Picman, 1986; Schmidt, 1999). Because the active site of STL is associated with features of the lactone ring, the stereochemistry of the junction between the lactone ring and the remaining compound has the potential to influence the biological activity of these compounds. Evidence of differential activity between *cis*- and *trans*-fused STL has been documented in laboratory assays (Beekman et al. 1997, Bodensieck et al. 2011, Takeda et al. 2011, Ahern Chapter 1). Importantly, two studies have shown that stereochemical variation in lactone ring junctions of STL found in *Xanthium strumarium* influences activity. Takeda et al (2011) demonstrated differential cytotoxicity of xanthatin and 8-epi-xanthatin against human cancer cell lines. In our lab, we have demonstrated differential feeding deterrence to grasshoppers (Ahern, Chapter 1). Further, field studies have shown that patterns of folivore damage are correlated with stereochemistry (Ahern, Chapter 2).

Distribution of *Xanthium strumarium* in Texas. *X. strumarium* is distributed throughout Texas, growing in pastures, riverbanks, roadsides, and other ruderal areas. It

occurs in isolated populations, ranging in size from a few individuals to thousands of plants (Ahern, pers. obs.). Monomorphic populations of plants producing *cis*-fused or *trans*-fused STL are found throughout eastern Texas, with a trend for plants producing *cis*-fused lactones to occur northeast of Houston and plants producing *trans*-fused lactones to occur to the south and west of the Houston area. (Figure 3.1).

Chemotyping of Plant Material. Individual source populations were chemotyped via reverse-phase high performance liquid chromatography (HPLC) with mass spectra and/or UV detection. Using our chromatographic conditions (described in detail in Chapter 2), *cis*-fused STL's elute earlier than their *trans*-fused diastereomers. Because no populations were found that did not contain either 8-epi-xanthatin (*cis*) or xanthatin (*trans*), we used these diastereomeric compounds to characterize populations; these compounds have characteristic retention times, UV, and MS spectra. The presence or absence of peaks of peaks corresponding to each of these compounds was used to classify each source population as *cis*, *trans*, or mixed. Mixed populations containing some individuals with *cis*-fused lactones and some individuals with *trans*-fused lactones do exist, but were rare in our sampling (one of 26 collections or <4%) and were not used in the experiments described below.

Planting Design. We selected sites throughout southeast and central Texas to span the range of habitats that *X. strumarium* typically inhabits (Ahern, pers. obs.). Site locations and characteristics are presented in Table 3.2. In order to prevent disturbance from large non-native mammals (cows & feral hogs), barbed wire fences were constructed around plots at four of the six sites where these animals were known to be present. Fences were not constructed at the remaining two sites due to the absence of

cows or feral hogs. Fences were designed not to exclude native deer, rabbits, or other mammals.

Mature burs were collected in the field in the fall of 2007, 2008, and 2009 from 19 sites in North America and Asia (Table 3.3). Individual seeds were excised from burs and germinated on moist tissue paper in the laboratory. Germinated seeds were transplanted into 3.8 x 10 cm Jiffy peat pellets, and grown in the greenhouse for approximately 2 weeks prior to planting in the field. Seedlings were transplanted at each site between May 21st and June 4th. Prior to planting, surrounding vegetation was mowed to reduce above ground competition during establishment. At each site, ten seedlings from each of 9 *cis* populations and 10 *trans* populations (totaling 190 seedlings) were planted in a grid with each plant 1.5 meters apart using a completely randomized design. Plants were hand watered over the first week after planting (1-3 times depending on local rainfall) to increase transplanting success.

Response variables. *Survival to Reproduction.* Over the course of the growing season, sites were periodically surveyed for plant survivorship to reproduction (i.e., to seed production). When possible, the most likely agent of mortality (i.e. drought, insect herbivory, mammalian herbivory) was recorded.

Folivore Damage. Early in the growing season (6/15-6/25) the percentage of leaf area removed by folivore insects was visually estimated for each leaf (excluding immature leaves smaller than ½ cm x ½ cm) on every plant. We focused on folivores because our previous research in this system has shown folivores impact high levels of damage on *X. strumarium*. Based on observations characteristic feeding damage by different insects, we have found that the majority of damage is caused by Acridid

grasshoppers, while limited damage is caused by katydids, caterpillars, beetles, and mollusks. To maintain consistency between plants and sites, all damage was estimated by a single observer. A composite measure of damage per plant was then made by dividing the sum of percentage area damaged per leaf by the total number of leaves.

Plant Fitness. *X. strumarium* primarily self pollinates (Dinelli et al. 2003), and amount of mass allocated to male flowers is minimal (<1%) in comparison to the mass allocated to female flowers and seeds (Sugiyama and Hirose 1991). Because of these reasons, we deemed that seed set would be the most appropriate metric of lifetime reproductive fitness. After plants naturally senesced, all burs were collected from each plant and counted in the laboratory. Material was sorted by hand to remove undeveloped, immature burs before counting. Mean bur mass varied dramatically across source populations (range: 95 mg - 566 mg) and bur mass was strongly positively correlated with seed mass ($r = 0.9438$, $P < 0.0001$, $n = 251$) (Ahern, unpublished). Seed mass is commonly positively associated with germination rate, seed longevity, seedling competitive ability, and seedling survival in numerous species (Silvertown 1989, Sadras 2007); furthermore, in *X. strumarium*, seed size is known to influence germination rate, with larger seeds germinating faster (Zimmerman and Weis 1983). Given the extensive variation in seed size found among different source populations of *Xanthium strumarium*, and the aforementioned considerations, it seems likely that seed size influences the reproductive value of seeds, and should thus be accounted for in an interpretation of fitness. Thus, to bracket the possibilities – from a) all seeds contribute equally to fitness regardless of size, to b) seeds with greater mass contribute more to fitness – we

performed all analyses on two indices of fitness; 1) bur number, and 2) the product of bur number by source population average bur mass, hereafter total bur mass.

Statistical Analyses. Unless specified otherwise, all analyses were performed in Proc Glimmix using SAS version 9.2, and investigated the effects of stereochemistry, site, and their interaction. Stereochemistry was treated as a fixed effect, and source population was treated as a random effect, nested within stereochemistry. As such, source population was the effective unit of replication. Site was treated as a fixed effect, as sites were chosen to represent a range of habitats which *X. strumarium* typically inhabits. If spatial variation in performance of the morphs is implicated in maintenance of the stereochemical trait polymorphism, we expect a significant site by stereochemistry interaction, such that each morph has superior performance (lower herbivory, higher fitness) in at least one site.

Does stereochemical variation affect folivore damage? Does this effect vary spatially? To assess whether variation in stereochemistry affects folivore damage, we examined the effects of stereochemistry, site, and their interaction on folivore damage. Since folivore damage is a proportional measure bounded between 0 and 1 (0% - 100%), we fit the data to a beta distribution with a logit link function. Our data included individual plants with scores at the extreme tails of these distributions, 0 and 1 (corresponding to 0% and 100% damage respectively). With a logit-link function, values of 0 and 1 result in undefined values of $-\infty$ and $+\infty$ respectively. To address this issue, we replaced 0 and 1 values with constants (0.0014 and 0.9986 respectively). This process is similar to the ‘additive replacement’ methodology used in compositional data analysis literature (Martín-Fernández and Thió-Henestrosa 2006), advocated for ecological use by

Warton et al. (2011). The chosen values are outside of the nearest extreme damage levels (0.142857% and 97.5%), and thus did not change the rank order of proportions in the dataset. Alternative replacement values and other methods of analysis were examined and did not qualitatively change the results.

Does folivore damage affect plant survival to reproduction? To examine the influence that folivore damage had on plant survival to reproduction (a binary variable), we performed a series of mixed model logistic regressions. We first used a full interactive model with folivore damage, site, and their interaction as fixed effects, source population nested within site and the folivore damage \times site interaction as a random effect. We fit this as a zero-intercept model to allow independent intercepts at each site. Stereochemistry was not incorporated into this model, as stereochemistry and folivore damage were correlated, and thus suffered from issues of multicollinearity. In order to perform significance tests and extract parameter estimates at each individual site, we then fit a similar model, excluding the main effect term of folivore damage, thus drawing independent parameter estimates and significance tests for each site. In this site specific model, we examined whether the relationship between folivore damage and survival to reproduction had a slope significantly different than zero, with negative slopes interpreted as evidence that herbivores negatively affect plant fitness.

Does stereochemical variation affect plant survival to reproduction? To examine the effect that stereochemistry had on plant survival, we fit a similar mixed model logistic regression analysis, here using stereochemistry, site, and their interaction as predictor variables.

Is there spatial variation in the identity of the most fit morph, such that each stereochemical morph performs best in some sites? To investigate whether there is spatial variation in the identity of the most fit morph, we examined the influence of stereochemistry, site, and their interaction on plant fitness in a generalized linear mixed model. Fitness here is defined as the total female reproductive output of a plant, and the analysis included both plants that survived to reproduction and those that did not. To bracket the potential contributions of seed mass to fitness (see discussion above and below), we ran independent models using bur number and total bur mass as response variables. Since fitness data was count-based, and the variance was not equal to the mean, we utilized a negative binomial distribution with a log link function. Model fit was compared to alternative distributions (Gaussian, Poisson, over-dispersed Poisson) using log-likelihood ratios. Due to extensive site-to-site variation in fitness, we observed substantial heteroscedasticity (in predicted residual plots, and via a significant site level effect in a Levine's test). Therefore, we present results from the main effect level analysis to examine the overall effects of stereochemistry, site, and their interaction. Subsequently, we ran individual analyses for each site to investigate the influence of stereochemistry at each site. Significant interactive effects between stereochemistry and site, where each morph has equal or higher fitness than the other morph, were interpreted as evidence of spatially variable selection.

Does the intensity of folivore attack predict the identity of the most fit morph across sites? To examine whether the overall intensity of folivore attack can predict the identity of the most fit morph all across sites, we examined how stereochemical variation and folivore damage influenced relative fitness. Because of extensive variation across

sites in mean fitness, we used relative fitness (raw bur number or raw total bur mass divided by the site mean for number or mass, respectively). We then performed regression analyses (one using each fitness metric) in Proc Mixed, using stereochemistry and folivore damage as predictor variables. As each source population was replicated at each site, we used source population and the stereochemistry \times site interaction as random effects in order to properly account for the non-independence of data points. We parameterized our model to fit separate intercepts and slopes for each morph (*cis* & *trans*). We then used adjusted means contrasts to conduct pair wise tests to examine whether either intercepts or slopes differed between morphs. Significant differences in intercepts were interpreted as evidence that the relative fitness of morphs differed in the absence of folivore damage. Significant differences in slopes were interpreted as evidence that morphs differed in their relative fitness response to folivore damage; e.g., a significant negative slope indicates that increasing levels of folivore attack are associated with reduced fitness of a morph (relative to the fitness of the alternate morph).

3.3 Results

Does stereochemical variation affect folivore damage? Does this effect vary spatially? Plant stereochemistry was associated with folivore damage, with plants producing *cis*-fused STL experienced more folivore damage than plants producing *trans*-fused STL ($F_{1,16} = 30.41$, $P < 0.0001$). The magnitude of folivore damage varied across sites, ranging from 6.1% to 80.6% leaf area consumed ($F_{4,64} = 196.09$, $P < 0.0001$). The effect of stereochemistry on folivore damage also varied spatially indicated by a significant interaction term ($F_{4,64} = 2.63$, $P = 0.0424$), where plants producing *cis*-fused STL experienced more folivore damage than plants producing *trans*-fused STL at four

sites, with no significant differences at a fifth site (Liberty; Figure 2a). As plants at Liberty experienced the lowest overall levels of folivore damage (mean 6.1% leaf area removed), this indicates that stereochemical variation influences folivore damage in a consistent fashion, except where folivore damage is low.

Does folivore damage affect plant survival to reproduction? Plant survival to reproduction was negatively correlated with percentage folivore damage ($F_{1,82} = 10.91$, $P = 0.0014$), suggesting that folivore damage reduces survival to reproduction. Individual sites differed in the proportion of plants that survived to reproduction ($F_{5,85} = 23.88$, $P > 0.0001$), but the relationship between folivore damage and survival was consistent at four of five sites (damage \times site interaction, $F_{4,82} = 0.70$, $P = 0.5931$; Figure 3). At the fifth site (Liberty), there was no relationship between folivore damage and survival to reproduction, presumably due to the low levels of damage at this site.

Does stereochemical variation affect plant survival to reproduction? Stereochemical variation influenced plant survival to reproduction at some sites, indicated by a significant main level effects of stereochemistry ($F_{1,17} = 10.93$, $P = 0.0042$) and site ($F_{4,68} = 44.89$, $P < 0.0001$), along with a significant interaction between stereochemistry and site ($F_{4,68} = 4.26$, $P = 0.0039$). At three sites, plants producing *trans*-fused STL had higher survival than plants producing *cis*-fused STL, while there were no differences at the remaining two sites (Figure 2b).

Is there spatial variation in the identity of the most fit morph, such that each stereochemical morph performs best in some sites? With one of our metrics of fitness, bur number, we found a significant stereochemistry \times site interaction ($F_{4,64} = 9.71$, $P < 0.0001$) and evidence that each morph has the highest fitness in alternate sites: plants

producing *trans*-fused STL had higher fitness at one site (Nixon), plants producing *cis*-fused STL had higher fitness at one site (Liberty), with no significant differences at the remaining three sites (Figure 4a). In contrast, when fitness was assessed via total bur mass, we again found a significant stereochemistry \times site interaction ($F_{4,64} = 9.50$, $P < 0.0001$), but found that plants producing *trans*-fused STL had higher fitness at three sites while there were no differences between morphs at the remaining two sites (Figure 4b). Therefore, depending on the relative contribution of seed mass versus seed number to offspring fitness, the evidence suggests that each morph can have the highest fitness in certain locations (if fitness is independent of seed mass) or that the *trans* form often has the highest fitness, with instances of equal fitness (if seed mass is as important to fitness as seed number). It seems likely that there is some contribution of seed mass to fitness, but probably is weaker than that captured by total bur mass here.

Does the intensity of folivore attack predict the identity of the most fit morph across sites? We found significant relationships between spatial variation in the intensity of folivore damage and the relative fitness of plants producing *cis*-fused or *trans*-fused STL (Figure 5). For bur number, we found a significantly negative relationship between folivore damage and relative fitness for *cis* plants ($F_{1,35} = 10.95$, $P = 0.0022$, Figure 5a), and a significantly positive relationship between folivore damage and relative fitness for *trans* plants ($F_{1,35} = 7.95$, $P = 0.0078$). Planned contrasts between *cis* and *trans* plants revealed significant differences between both intercepts ($F_{1,16} = 6.25$, $P = 0.0237$) (suggesting different costs of producing the defense in the absence of herbivores) and slopes ($F_{1,70} = 18.39$, $P < 0.0001$) (suggesting different benefits of producing the defense in the presence of herbivores). For total bur mass, we found a significantly negative

relationship between folivore damage and relative fitness for *cis* plants ($F_{1,35} = 5.06$, $P = 0.0311$), and a non-significant positive trend for *trans* plants ($F_{1,35} = 3.03$, $P = 0.0901$, Figure 5b). Planned contrasts showed that *cis* and *trans* plants had significantly different slopes ($F_{1,70} = 7.72$, $P = 0.0070$), with no difference in intercepts ($F_{1,16} = 1.02$, $P = 0.3282$). These results indicate that as the intensity of folivore damage increases, plants producing *cis*-fused STL perform comparatively worse than plants producing *trans*-fused STL. At no/low levels of folivore damage, the results differed between our two fitness metrics. With fitness estimated as bur number, significantly different intercepts suggests that in the absence of folivore damage, *cis* plants have higher relative fitness than *trans* plants (Figure 5a). In contrast, with fitness estimated as total bur mass, the morphs did not have significantly different intercepts, suggesting that in the absence of folivore damage, *cis* and *trans* plants have equal fitness (Figure 5b). It is likely that the true fitness metric lies somewhere between these two brackets.

3.4 Discussion

Our results provide conclusive evidence that spatial variation in the intensity of herbivory imposes spatially variable selection on the sesquiterpene lactone stereochemical trait polymorphism in *X. strumarium*. The key result is that variation in the magnitude of folivore damage resulted in genotype-by-environment (GxE) interactions, such that, each stereochemical morph had superior performance in at least one location (or equal performance using total bur mass as the fitness measure). Importantly, this is one of only two studies of which we are aware to show that chemical variation in defensive compounds can be maintained by spatially varying selection (see also *Thymus* examples, Table 3.1), and to our knowledge, is the first to implicate

herbivores as agents of selection. This conclusion is supported by a number of individual results. First, we found spatial variation in the intensity and specificity of folivore damage, such that plants producing *cis*-fused STL experienced more folivore damage than plants producing *trans*-fused STL at some sites. As folivores were hypothesized to be a selective agent acting upon the *cis/trans* defensive polymorphism in *X. strumarium*, spatial variation in the intensity of folivore damage indicated that the strength of selection may also vary spatially. Second, folivorous insects were a potent selective force in this system, since folivore damage was correlated with survival to reproduction, bur production, and stereochemistry (Figures 3.2 & 3.3), resulting in correlations between stereochemistry and plant fitness (Figure 3.4). Site-specific variation in these correlations indicated that folivorous insects impose spatially variable selection on the *X. strumarium* polymorphism. Third, the effects of pre-reproduction mortality persisted to influence the average reproductive fitness of *cis*- and *trans*-fused STL morphs (Figure 3.4). Although the outcome of our analyses depended upon the metric of fitness used (bur number or total bur mass, as discussed below), both analyses resulted in significant GxE interactions, the signature of spatially variable selection. Finally, the intensity of folivore damage at a site was sufficient to predict which morph had superior performance, with *trans* plants having higher relative fitness in sites experiencing high levels of folivore damage, while *cis* plants had equal or higher relative fitness where folivore damage was low (depending upon the fitness metric utilized, Figure 3.5).

The choice of a fitness metric and its consequences for interpretations of polymorphism maintenance Analyses of site specific fitness patterns indicate that depending upon the fitness metric utilized (bur number or total bur mass) either each

morph can have higher fitness in different places (bur number) or that *trans* plants outperform *cis* plants at some sites, while they have equivalent fitness at others (total bur mass). This indicates that the fitness significance of bur size, and hence seed size, may play an important role in our understanding of the maintenance of this stereochemical trait polymorphism. Studies investigating the paradox of seed size:seed number relationships have indicated that often seed size is correlated with dispersal ability, seed predation, germination, competitive ability, biotic and abiotic tolerances, etc (Silvertown 1989, Moles and Westoby 2004a, 2004b, 2006). Each of these factors contributes to the net reproductive output of a given plant, both individually and interactively (Moles and Westoby 2004a, 2006). In *X. strumarium*, bur size has been shown to correlate positively with germination percentage and germination rate (Zimmerman and Weis 1983), and negatively with susceptibility to seed predators (Hare and Futuyma 1978), indicating a superiority of larger burs. However, bur size can be negatively correlated with growth rate (Zimmerman and Weis 1983) and number of burs produced (Farris and Lechowicz 1990), indicating the possible advantages of smaller fruits. Further, the relationships between these factors and bur size are known to be environmentally dependent (Hare 1980, Lechowicz and Blais 1988), and thus the benefits and costs of different bur sizes can only be understood in the context of the recipient community. Measuring each potential fitness component and assessing their relative importance across a range of environments was beyond the scope of this work, as this would require following the dispersal and recruitment of burs from each source population at every site through (at minimum) the following year. Interpreting fitness patterns based on seed number provide clear evidence for this, as each morph has superior fitness in different environments.

Although we did not detect evidence that *cis*-plants had higher fitness at any sites when interpreting fitness based on total bur mass, it is likely that this pattern would emerge if plants were tested across a larger range of environments and if we had accurate and site-specific estimates of the contribution of bur size to plant fitness.

Associations between STL stereochemistry and seed size. We observed a strong association between STL stereochemistry and seed size/seed number, with *cis* plants producing numerous small seeds and *trans* plants producing fewer, larger seeds. Several hypotheses may account for this association. First, pleiotropy between STL stereochemistry and seed size could lead to the observed associations. Based on our current understanding of STL biosynthesis, this polymorphism appears to be caused by allelic variation in a single gene encoding a cytochrome P450 enzyme responsible for the stereospecific formation of the STL lactone ring junction (D. K. Ro, pers. comm., Ikezawa et al. 2011). Given the highly specialized function and substrate specificity observed in P450 enzymes, it seems unlikely that allelic variation in this gene would have pleiotropic effects on seed/bur size which are presumably encoded by an entirely separate suite of genes.

Second, the association between lactone ring stereochemistry and seed/bur size may reflect the correlated evolution of traits in lineages experiencing different environments. It has been proposed that variation in a trait related to environmental stress can lead to adaptive changes in a suite of other traits correlated with specific environments, favoring different trait combinations in different environments (Chapin III et al. 1993). Since different morphs are often found in spatially separated monomorphic populations, selection would not theoretically predict that a morph would go extinct if the

other is absent (i.e. out competed). In monomorphic populations, there is no heritable variation in stereochemistry (as there is only 1 morph), and thus selection cannot drive change in that trait (although selection can act on other quantitative and qualitative variation in STL). However, selection is expected to act on other traits that increase fitness in those environments; e.g., seed size. Such a situation could have potentially led to different trait adaptive syndromes, where plants producing *cis*- or *trans*-fused STL have evolved different suites of traits that make each morph adapted to different environmental conditions. For example, plants producing *cis*-fused STL, which confer less resistance to folivorous insects, may have evolved to occupy habitats where folivore damage is infrequent or minimal. If such habitats tend to be “open” areas of low plant density, low competition, and high resource availability, as suggested by (Blais 1984, Lechowicz and Blais 1988) selection could have driven the evolution of smaller, more numerous seeds, which are hypothesized to be adaptive in these situations (Geritz 1995, Geritz et al. 1999). Alternatively, plants producing *trans*-fused STL, which confer more resistance to folivorous insects, may have evolved to occupy habitats with where folivore damage or the ecological cost of folivore damage is higher. If these habitats were also associated with higher competition or lower resource availability, large seeds could be more adaptive. In these conjectures are correct, this could have led to the observed correlations between stereochemistry and seed size. It would be intriguing to investigate whether STL stereochemistry is correlated with different habitat types, and whether associated trait differentiation between morphs corresponds to this.

Preliminary data provide some support for this hypothesis. Although the authors did not characterize STL stereochemistry of plants, Lechowicz and Blais found different

seed sizes were associated with different habitats in *X. strumarium*, with small seeded individuals found along riverbanks and large seeded individuals found along roadsides and waste areas (Blais 1984, Blais and Lechowicz 1989), suggesting habitat-associated trait syndromes may exist in this system. However, fine scale mapping of habitat associations of different morphs and seed sizes, along with environmental characteristics of different habitats would be necessary to examine this. In order to understand whether seed size and stereochemistry have evolved in tandem in response to environmental conditions, one could experimentally disentangle these factors. This could be accomplished by generating recombinant inbred lines, to break up correlations between stereochemistry and seed size. Examining the performance of different lines in a range of habitats could provide insight into the individual and interactive effects that each component contributes to reproductive success in each habitat.

Do *trans*-fused STL always confer superior resistance against plant enemies?

At this point, our evidence suggests that *trans*-fused STL confer more resistance against folivorous insects (predominantly Orthopterans) than *cis*-fused lactones. In that respect, it appears as though herbivore-mediated selection is unilateral: in the presence of adequate selective pressure from folivorous insects, plants with *trans*-fused STL should outperform plants with *cis*-fused STL. However, sesquiterpene lactones exhibit a range of activities against a diverse group of organisms, spanning bacteria, protists, fungi, plants, invertebrates, and mammals (Rodriguez et al. 1976, Picman 1986). Despite a limited group of functional groups and modes of activity, these compounds exhibit diverse structure activity relationships (Schmidt 1999, 2006). Thus one can envision a case where *cis*-fused STL confer more resistance than *trans*-fused STL against a different

group of plant enemies than the folivorous insects examined here. The existing evidence that documents differential bioactivity between *cis*- and *trans*-fused STL is limited at best, consisting of four studies to date in our knowledge (Beekman et al. 1997, Bodensieck et al. 2011, Takeda et al. 2011, Ahern Chapter 1). From this work, lactone junction stereochemistry has been implicated in differential bioactivities of *cis*- and *trans*-fused STL against insects (preference) and various mammalian cell lines. Three of these studies demonstrate the pattern observed in the field in this study (*trans*-fused lactones confer higher activity); while one shows that *cis*-fused lactones confer higher activity. Albeit limited to a small set of compounds and assays, this evidence suggests that *cis*-fused STL could possibly confer more resistance than *trans*-fused STL against a different plant enemy. Field tests are badly needed, particularly of non-arthropod and non-mammalian enemies such as plant-pathogenic bacteria and fungi.

A role for herbivore-mediated spatially varying selection in the maintenance of variation in plant defensive compounds. Evolutionary theory predicts that spatially variable selection can provide conditions for the maintenance of a genetic polymorphism (Levine 1953, Hedrick 1986, Hedrick 1998, Byers 2005). The results of this study indicate that the STL stereochemical trait polymorphism in *X. strumarium* could be maintained via spatially variable selection, as indicated by significant GxE interactions in fitness (figure 5). Our evidence suggests that selection on different stereochemical morphs was imposed by folivorous insects, and the strength of this selection was partially dependent upon the intensity of folivory in an environment (figure 6). To the best of our knowledge, we believe this is the first study to date to show that spatially variable selection imposed by herbivores contributes to the maintenance of a defensive chemical

trait polymorphism. In contrast, other studies have indicated that herbivores could be involved in the maintenance, but spatially variable selection imposed by abiotic stress played a larger role. For example, Thompson and colleagues have investigated the maintenance of distinct monoterpene chemotypes of *Thymus vulgaris* in Southern France. Although they have found the different chemotypes are correlated with preference of various herbivores (Linhart & Thompson 1999), summer drought and early-winter freezing mortality have been implicated as the putative causal agents behind the spatial distribution of phenolic and non-phenolic chemotypes of this species (Amiot et al 2005, Thompson et al 2007). Thus, although the putative role of monoterpenes in *Thymus vulgaris* is defensive, apparently variation in this trait may be maintained via pleiotropic effects on climatic tolerances. This may in fact be a more general trend in polymorphic traits, where the agents of selection maintaining polymorphisms are often not the ‘obvious’ agents suggested by the putative function of the trait. For example, Whitney & Stanton (2004) found that Hemipteran seed predators imposed strong selection on fruit color in *Acacia ligulata*, despite the fact that the polymorphic trait (aril color) was not visually apparent at the time of damage and the fact that avian seed dispersers would be the expected selective agent.

Conclusions. Variable selection is commonly proposed as a mechanism maintaining polymorphic traits in nature. We found that spatial variation between sites resulted in GxE interactions, indicating STL lactone ring stereochemical variation is an evolutionarily balanced polymorphism in *X. strumarium*. Fitness patterns were correlated with the intensity of folivore damage at each site; implicating herbivores as the selective agent responsible for the maintenance of this polymorphic trait. This study provides key

evidence that herbivore mediated spatially variable selection likely plays a role in the maintenance of defensive chemical polymorphic traits.

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3.7 Tables

Species	<i>Barbarea vulgaris</i>	<i>Boechera stricta</i>	<i>Thymus vulgaris</i>	<i>Datura wrightii</i>	<i>Lotus corniculatus</i>	<i>Trifolium repens</i>
chemical trait	Glu	Glu	Mon	Tri	CY	CY
type of polymorphism	Q/Q	Q/Q	Q/Q	(+/-)	(+/-)	(+/-)
heritable variation in trait	y	y	y	y	y	y
variation in field damage	y	y	y	y	y	y
fitness quantified in field	-	-	y	y	y	y
selective basis of polymorphism	-	-	y*	-	-	-
proposed mechanism	VS	VS	VS	VS	VS	VS

Table 3.1) Representative defensive chemical trait polymorphisms along with relevant information for documenting a selectively maintained plant defensive chemical trait polymorphism. Defense type: Glu=glucosinolate, Mon=Monoterpene, Tri=Trichome type, PrI=Protease inhibitor, CY=Cyanogenesis. Polymorphism types: (+/-) = presence/absence, Q/Q = qualitative, where morphs differ in the relative abundance of different compounds. We assume that the proposed mechanism is variable selection (VS) in each case. Representative citations for each system are as follows. *Barbarea vulgaris*: (van Leur et al. 2006, Van Leur 2008, van Leur et al. 2008a, van Leur et al. 2008b). *Boechera stricta* (Schranz et al. 2009, Manzaneda et al. 2010). *Thymus vulgaris* (Thompson et al. 1998, Linhart and Thompson 1999, Thompson et al. 2003, Amiot et al. 2005, Linhart et al. 2005, Thompson et al. 2007). *Datura wrightii* (Elle et al. 1999, van Dam et al. 1999, Elle and Hare 2000, Hare and Elle 2002, Hare et al. 2003, Hare and Elle 2004, Hare and Smith 2005). *Lotus corniculatus* (Jones 1962, Jones 1966, 1970, Compton et al. 1983). *Trifolium repens* (Daday 1965, Ennos 1981, Dirzo and Harper 1982, Hughes 1991, Saucy et al. 1999, Olsen et al. 2008, Olsen and Ungerer 2008)

Table 3.2. Locations and site characteristics of experimental gardens used in this study. Soil characteristics were obtained from the National Resources Conservation Service soil survey.

Site	Latitude	Longitude	Soil Type	Dominant Groundcover	Vegetational Area
Coldspring	30° 34' 47" N	95° 7' 12" W	Fine sandy loam	grasses	Piney Woods
JHWMA	28° 55' 58" N	95° 26' 12" W	Silty clay loam	forbs	Gulf Prairies and Marshes
Liberty	30° 3' 23" N	94° 49' 53" W	Clay	forbs	Gulf Prairies and Marshes
Nixon	29° 20' 45" N	97° 43' 39" W	Fine sandy loam	grasses	Post Oak Savannah
Warren Ranch	29° 58' 35" N	95° 51' 33" W	Fine sandy loam	grasses	Post Oak Savannah

Table 3.3. Names and collection locations of seed sources used in experimental gardens.

ID	Site of Seed Collection	State	Country	Latitude	Longitude
<i>Cis</i>					
ANF	Angelina NF	Texas	USA	31°02'34" N	94°17'33" W
CYC	Cypress Creek	Texas	USA	29°57'57" N	95°36'25" W
GUA	Guangzhou	Guangdong	China	23°11'11" N	113°21'38" E
JEJ	Jessie Jones	Texas	USA	30°01'52" N	95°18'9" W
KPC	Katy Prairie Conservancy	Texas	USA	29°56'06" N	95°55'48" W
LAC	Lake Creek	Texas	USA	30°16'48" N	95°42'19" W
NC	Inglis Farm	North Carolina	USA	36°04'13" N	76°26'49" W
TEN	Tennessee	Tennessee	USA	36°23'50" N	83°27'28" W
WUH	Wuhan	Hubei	China	30°32'37" N	114°24'49" E
<i>Trans</i>					
AUS	Austin	Texas	USA	30°11'51" N	97°37'51" W
BRR	Brazos River	Texas	USA	29°34'3" N	95°48'35" W
FTS	Ft Stockton	Texas	USA	30°53'33" N	102°51'47" W
GOL	Goliad	Texas	USA	28°42'4" N	97°23'42" W
HCC	Houston Coastal Center	Texas	USA	29°23'27" N	95°02'34" W
JAC	Cozy Corner	Texas	USA	29°51'18" N	96°50'34" W
WRR	Warren Ranch	Texas	USA	29°58'42" N	95°51'01" W
COL	Colorado	Colorado	USA	38°45'01" N	107°58'55" W
DG	Columbus	Texas	USA	29°41'30" N	95°33'02" W
NIX	Nixon	Texas	USA	29°20'45" N	95°43'39" W

3.8 Figures

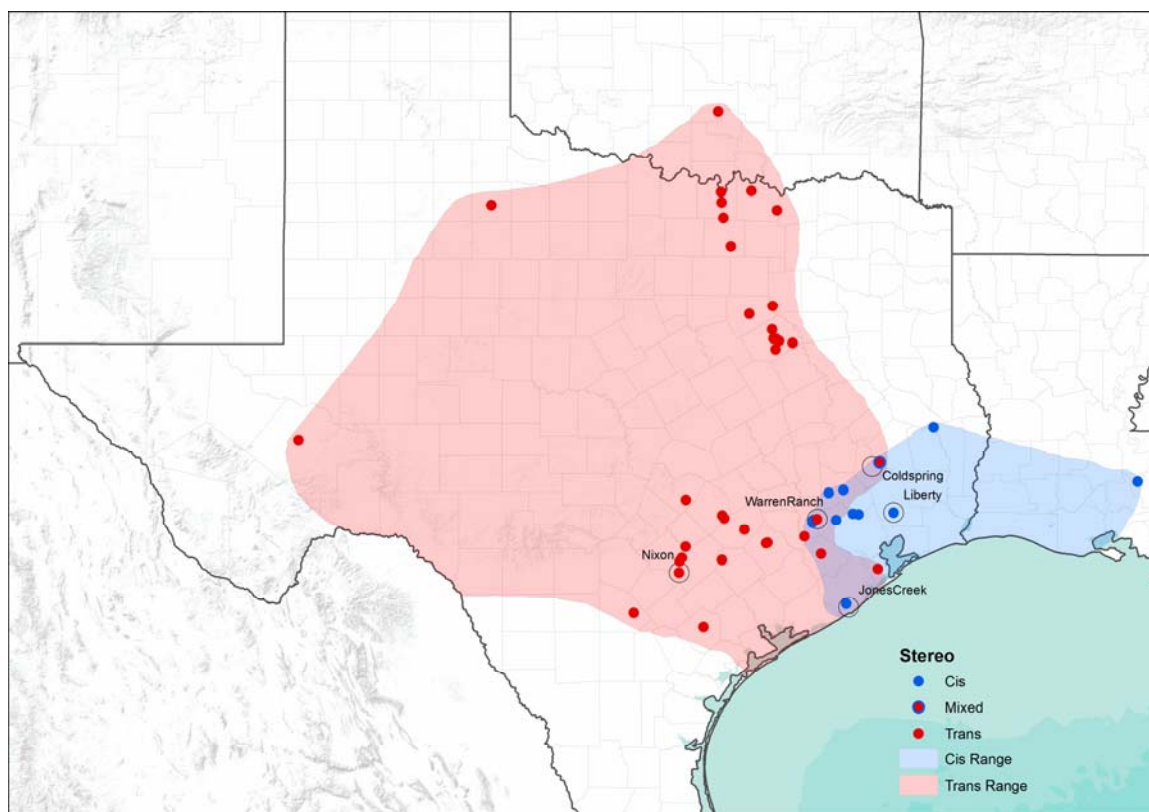


Figure 3.1. Distribution of *Xanthium strumarium* in Texas. Open circles denote locations of experimental gardens. Red circles denote populations of plants producing all *trans*-fused sesquiterpene lactones. Blue circles denote populations of plants producing all *cis*-fused sesquiterpene lactones. One population with red and blue had plants producing both *cis*- and *trans*-fused sesquiterpene lactones, along with plants producing all *cis*-fused or all *trans*-fused sesquiterpene lactones.

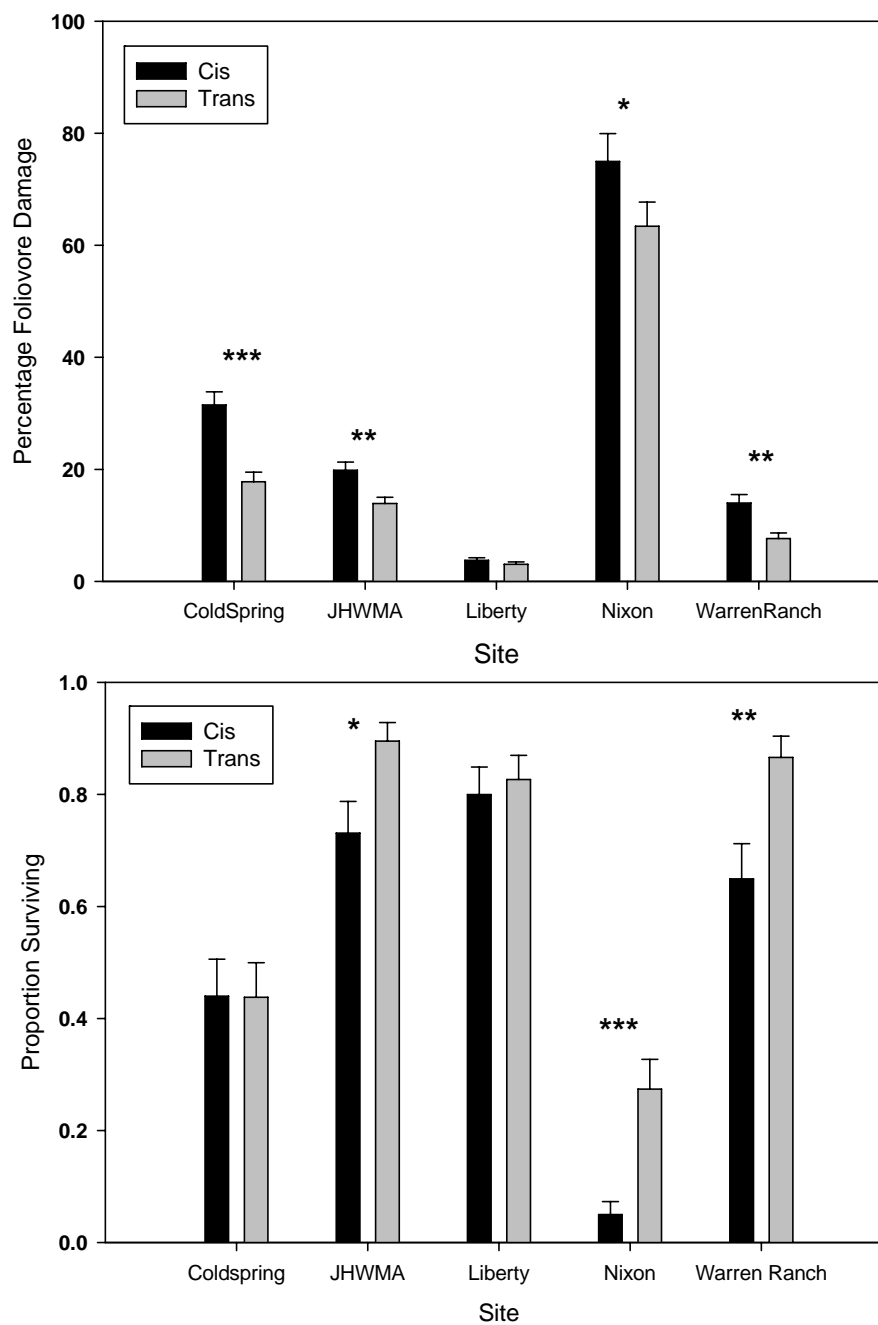


Figure 3.2. a) Stereochemistry influenced percentage chewing damage at four of 5 sites, with *cis* morphs experiencing more damage than *trans* morphs. Stereochemistry, site, and their interaction influenced the proportion of plants surviving to reproduction. Data are presented as least squares means and standard errors. Asterisks indicate statistical significance (* $\alpha=0.05$, ** $\alpha= 0.005$, *** $\alpha=0.0005$)

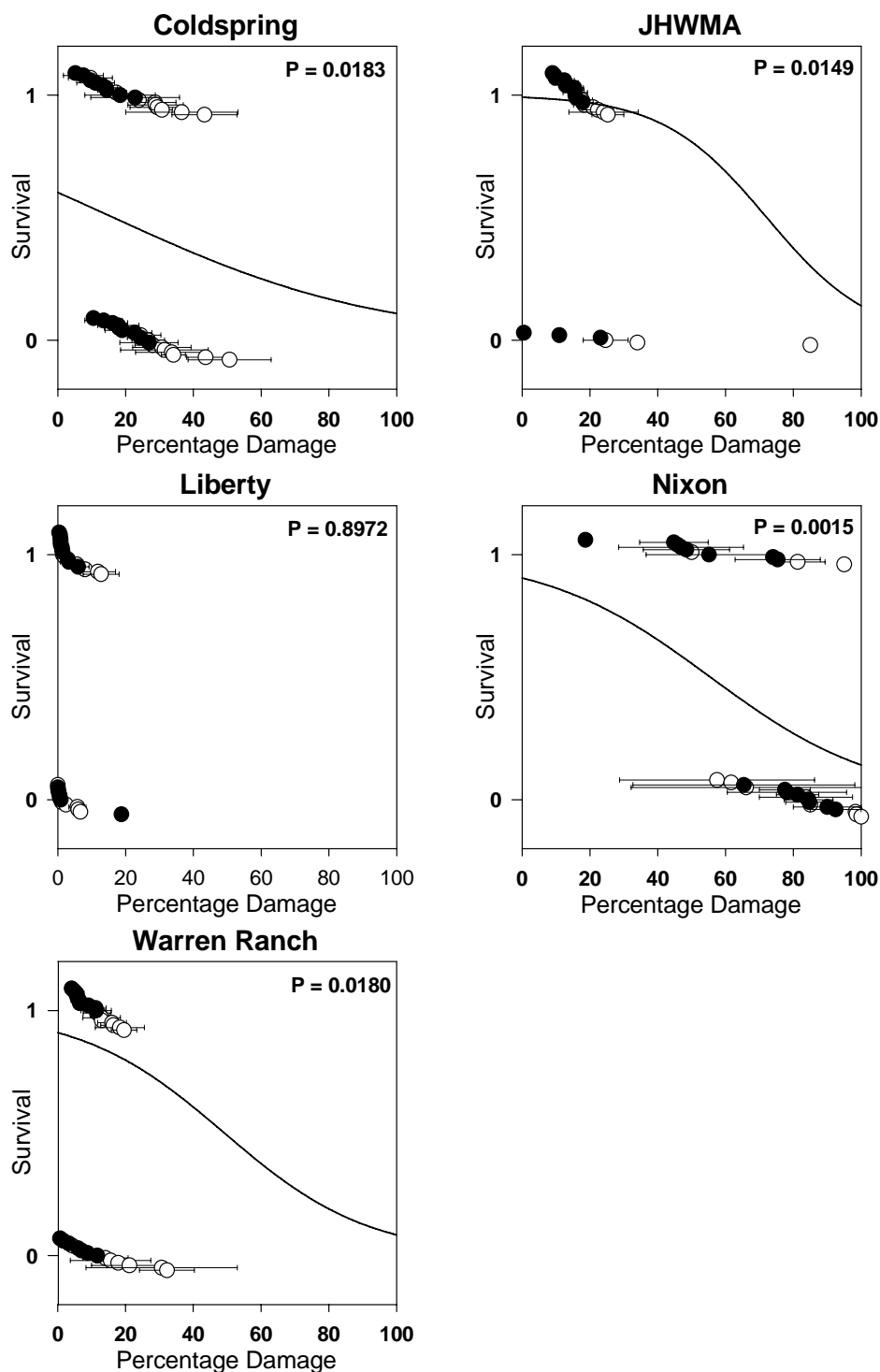


Figure 3.3: Folivore damage was significantly negatively correlated with survival to reproduction at Four of Five Sites. Each point presents the mean chewing damage and standard error for plants in a source population that survived to reproduction (1) or did not survive to reproduction (0). Points are offset off of the 0 and 1 lines for illustrative purposes only. P-values indicate a significantly negative slope parameter.

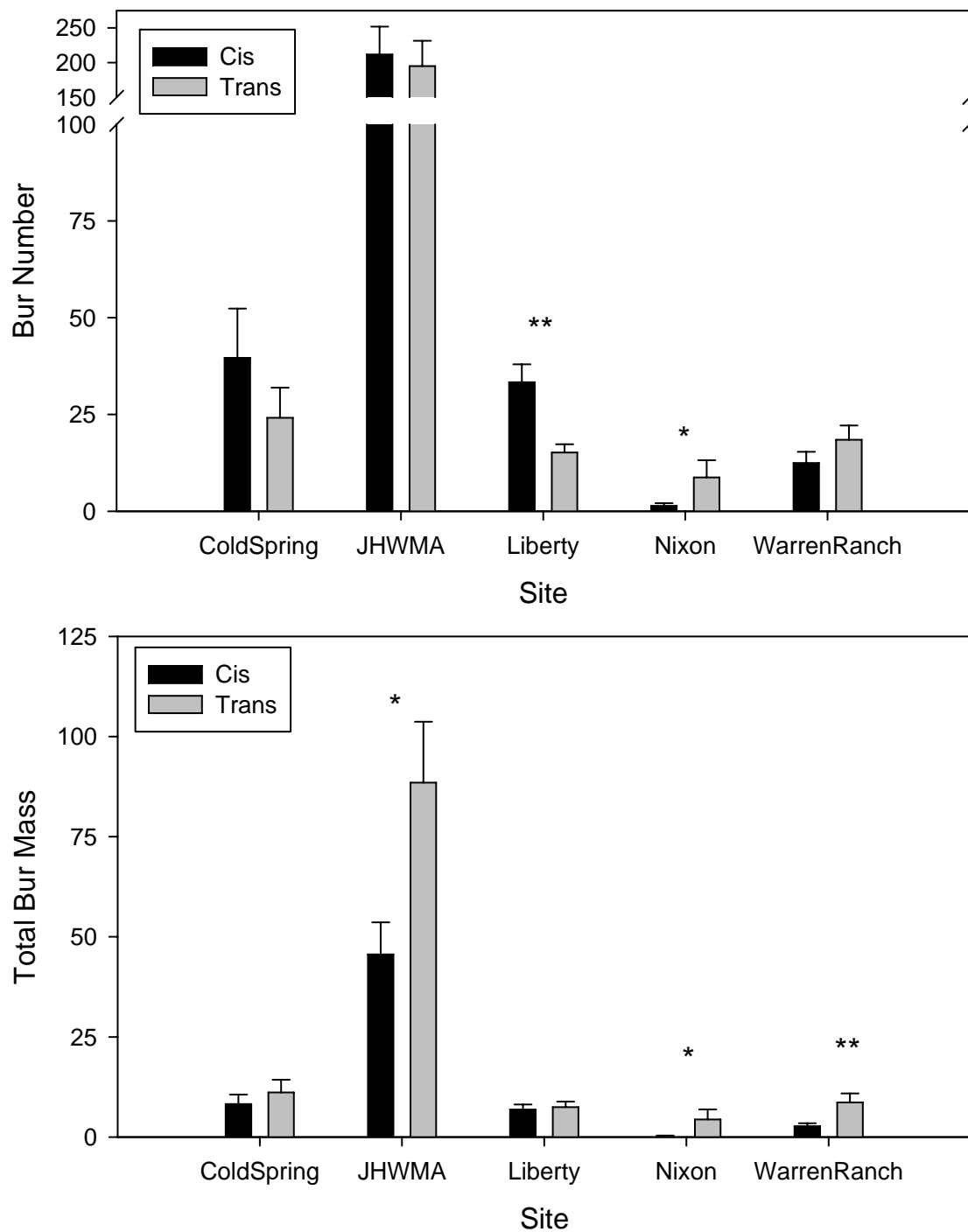


Figure 3.4: Reproductive fitness varied between morphs and across sites. Data are presented as least squares means and standard errors. Asterisks indicate statistical significance (* $\alpha=0.05$, ** $\alpha=0.005$)

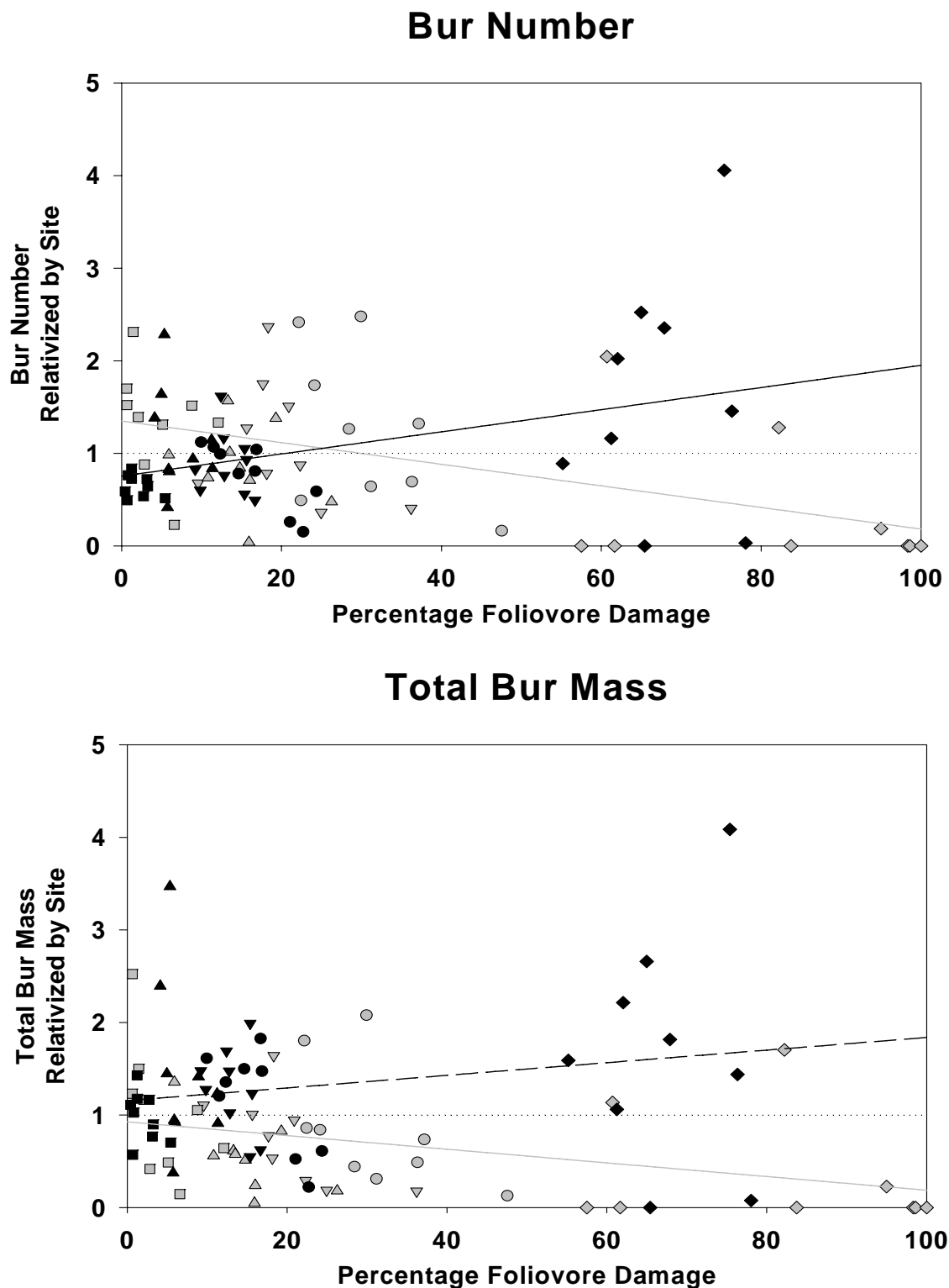


Figure 3.5: Spatial variation in the intensity of folivore damage predicts the relative performance of *cis* and *trans* morphs. Gray symbols signify *cis*-plants, while black symbols signify *trans* plants. Different shapes indicate different sites. Each point represents the mean value of an individual source population at each site.