

Sesquiterpene lactone stereochemistry influences herbivore resistance and plant fitness in the field

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- **Background and Aims** 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.
- **Methods** Natural variation in sesquiterpene lactones of the common cocklebur, *Xanthium strumarium* (Asteraceae), was used in conjunction with a series of common garden experiments to examine relationships between stereochemical variation, herbivore damage and plant fitness.
- **Key Results** The stereochemistry of sesquiterpene lactone ring junctions helped to explain variation in plant herbivore resistance. Plants producing *cis*-fused sesquiterpene lactones experienced significantly higher damage than plants producing *trans*-fused sesquiterpene lactones. Experiments manipulating herbivore damage above and below ambient levels found that herbivore damage was negatively correlated with plant fitness. This pattern translated into significant fitness differences between chemotypes under ambient levels of herbivore attack, but not when attack was experimentally reduced via pesticide.
- **Conclusions** To our knowledge, this work represents only the second study to examine sesquiterpene lactones as defensive compounds in the field, the first to document herbivore-mediated natural selection on sesquiterpene lactone variation and the first to investigate the ecological significance of the stereochemistry of the lactone ring junction. The results indicate that subtle differences in stereochemistry may be a major determinant of the protective role of secondary metabolites and thus of plant fitness. As stereochemical variation is widespread in many groups of secondary metabolites, these findings suggest the possibility of dynamic evolutionary histories within the Asteraceae and other plant families showing extensive stereochemical variation.

Key words: Asteraceae, common garden, defence, herbivory, plant–insect interactions, plant resistance, sesquiterpene lactone, *Xanthium strumarium*, common cocklebur.

INTRODUCTION

Linking plant secondary metabolites with 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 (Shonk 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, for example 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 and 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 behaviour 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. Clausen *et al.*, 1990; Stipanovic *et al.*, 2006) and alkaloids (e.g. Krug and Proksch, 1993). These laboratory bioassays clearly demonstrate that stereochemical variation in plant defensive chemicals can influence insect feeding behaviour and dietary preference, and therefore has the potential to be relevant in a field setting.

While 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 defence. 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 defence (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 defences 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.

Sesquiterpene lactones (hereafter STLs) are a diverse class of terpenoids with close to 5000 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 STLs are biologically active against bacteria, fungi, invertebrates and vertebrates (Rodríguez *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 STLs, 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).

A key feature of STLs 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 (Fig. 1). Hereafter, we refer to these as *cis*- and *trans*-fused STLs. Both *cis*- and *trans*-fused forms are found in angiosperms

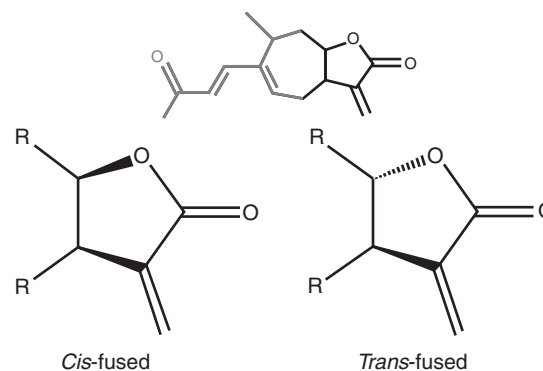


FIG. 1. In *Xanthium strumarium*, plants differ in the stereochemistry of the lactone ring junction. Pictured on the left is the lactone moiety of a *cis*-fused lactone ring, and on the right is that of a *trans*-fused lactone ring. At the top of the figure is the base skeleton (shown without stereochemical differences) of two common xanthanolide-type STLs found in *X. strumarium*, 8-epi-xanthatin (*cis*) and xanthatin (*trans*).

(based on data presented in Seaman, 1982; Budesínský and Saman, 1995). We have recently shown that this stereochemical variation in individually isolated STLs influences grasshopper feeding deterrence in laboratory assays (Ahern and Whitney, 2013). However, the importance of STL stereochemical variation in plant populations under field conditions has yet to be examined.

Here, we investigated the link between stereochemical variation in STLs and herbivore resistance in the field, using *Xanthium strumarium* (Asteraceae) as a study system. *X. strumarium* is polymorphic in STL ring junction stereochemistry, with an individual plant typically producing only *cis*- or only *trans*-fused lactones (Fig. 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 STL stereochemistry associated with herbivore resistance? (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 replicated common garden approach in south-east Texas, USA.

MATERIALS AND METHODS

Study species

Xanthium strumarium, the common cocklebur, is a cosmopolitan annual plant that inhabits ruderal habitats (Weaver and Lechowicz, 1983). The breeding system is dominated by selfing and 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. Strother, 2006). 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 STLs, occurring in concentrations in excess of 3 % dry weight (Chavez, 1973). Only 4–6 individual STLs have typically been reported in a given population of plants, although >40 different STLs 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 (Fig. 1). *X. strumarium* is polymorphic, in that within the species as a whole both *cis*- and *trans*-fused lactones are found, while most natural populations are monomorphic (all of the plants produce either all *cis*-fused or all *trans*-fused STLs) (McMillan *et al.*, 1975a, b). Stereospecific ring formation is enzymatically controlled (Ikezawa *et al.*, 2011), and it has been hypothesized that the formation of *cis*- and *trans*-fused STLs in *X. strumarium* is caused by closely related orthologous or paralogous enzymes (D. K. Ro, University of Calgary, Calgary, AB, Canada, pers. comm.). This reaction occurs early in the biosynthesis of STLs, which subsequently undergo substantial modification, leading to numerous end products (Fischer *et al.*, 1979).

Plant material and chemotyping

Mature burs were collected in the field in autumn 2007 and 2008 from 19 sites in North America and Asia (Table 1). Individual seeds were excised from burs and germinated on moist tissue paper in the laboratory. Germinated seeds were transplanted into 3.8 × 10-cm Jiffy peat pellets, and grown in the greenhouse for approximately 2 weeks prior to planting in the field.

Individual source populations were chemotyped via reversed-phase high-performance liquid chromatography with mass spectral and/or UV detection. Here the experimental methods are

described in brief; full details are provided in the Supplementary Data. STLs were extracted from dry leaf material (collected from five or more plants per population) in chloroform, dried down *in vacuo*, dissolved in acetonitrile and filtered with a 5- μ m syringe filter. Populations were classified as producing either *cis*- or *trans*-fused STLs (or mixed) based on the presence of 8-*epi*-xanthatin (*cis*) or its diastereoisomer xanthatin (*trans*), determined by comparison of retention times, and UV and mass spectrometry spectra. These compounds were selected as references because they were present in all sample collections and could be unambiguously identified based on their retention times and UV absorbance maxima at 280 nm. Because the stereospecific lactone ring formation is believed to occur prior to subsequent structural modifications (Fischer *et al.*, 1979; Ikezawa *et al.*, 2011), it was assumed that the presence of one of these diastereoisomers reflected the stereochemistry of all STLs in a given sample. For more detail and discussion about this assumption and supporting evidence, see the Supplementary Data. Due to lack of commercially available reference standards and the large diversity of STLs found within and between samples, quantitative measurements were not attempted.

Common garden sites

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

TABLE 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	Texas	USA	30°01'52"N	95°18'9"W	x	x
KPC	Katy Prairie 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	Texas	USA	28°42'4"N	97°23'42"W	x	x
HCC	Houston Coastal Center	Texas	USA	29°23'27"N	95°02'34"W	x	x
JAC	Cozy Corner	Texas	USA	29°51'18"N	96°50'34"W	x	x
RAR	Recurrent 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

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 (totalling 400 seedlings) were planted in a completely randomized design. Plants were arranged in a grid spaced 2 m apart and were watered every 2–3 d for 1 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 (aimed at reducing the spread of this weedy plant), we were required to harvest plants prior to bur drop, and thus were unable to measure plant fitness in this first experiment.

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, 12 seedlings from each of 19 populations (11 *cis* and eight *trans*) were planted in a completely randomized design (totalling 228 per site, 456 total). Plants were arranged in a grid with 1.5 m spacing. After planting, we watered for 1 week to increase establishment success. Due to extreme drought conditions in this year, 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 and homogenize above-ground competition. Because our experimental design utilized source populations as replicates, the impact of herbivores on plant fitness was confounded with differences between populations in herbivore resistance. To de-confound these factors, in Experiment 2 we experimentally manipulated foliar damage above and below ambient levels. 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 2 weeks thereafter. Plants in the elevated damage treatment were mechanically damaged monthly starting in July by clipping off half of each leaf with scissors (KPC: 22 July, 19 August, 18 September and 17 October; HCC: 17 July, 16 August, 13 September and 10 October). This level of damage (50 % leaf removal) is within the upper range of damage naturally observed in the field (J. R. Ahern, unpubl. data). Herbivore damage (see below) was surveyed three times for each plant (KPC: 22 July, 19 August and 18 September; HCC: 17 July, 15–16 August and 10–12 September). Mechanical damage treatments were applied after each herbivore damage survey (with no mechanical damage prior to the first survey), and plants then grew new leaves before the subsequent survey. As a result, the measured damage levels of mechanically damaged plants reported below were less than the treatment applied (50 % removal).

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. The most abundant herbivores observed on plants were grasshoppers (Acrididae), particularly different *Melanoplus* species (e.g. *M. sanguinipes*, *M. femurrubrum*). At lower abundance and frequency, we also observed katydids (Tettigoniidae), flea beetles (Galerucinae) and various unidentified Lepidoptera. 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 (see Whitney *et al.*, 2006):

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

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. *C₂* = 15.05) and *n* is the total number of leaves scored per plant across all surveys (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 % 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 mass of an individual bur varied dramatically across source populations (range: 95–566 mg) and bur mass was strongly positively correlated with seed mass (*r* = 0.9438, *P* < 0.01, *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.

Statistical analysis

All analyses were carried out in SAS version 9.3 (SAS Institute, 2010). Response variables were log₁₀ transformed to achieve normality of residuals. Parameter estimates were

back-transformed to obtain values presented in the figures. 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. Because the methodology for measuring leaf damage varied across years, and response variables had different variance and covariance structures within each site, the three site/year combinations were analysed 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 ($D_{\text{holes}} + D_{\text{window}}$) was the response variable, source population was treated as a fixed factor and individual plants were the units of replication. For the two 2009 gardens, we excluded pesticide-treated and mechanically damaged plants, and investigated heritability using only plants with natural levels of leaf damage. We interpret significant effects of source population on herbivore resistance as evidence consistent with a heritable component of resistance. We note, however, that maternal effects from the source population environment could also contribute to such effects.

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 determine how much of the variation in herbivore damage is attributable to stereochemistry, we calculated Pseudo R_e^2 and Pseudo R_0^2 values from the covariance parameter estimates of the final model and those from reduced models (Singer and Willett, 2003). Pseudo R_e^2 values represent the proportional reduction in residual error variance between the fully fitted model and the null model with no predictor variables (Singer and Willett, 2003). Pseudo R_e^2 values are roughly analogous to traditional R^2 values from generalized linear models, and indicate the proportion of the total variance explained by the model. Pseudo R_0^2 values represent the proportional reduction in level-1 residual variance (in this case, reduction in the covariance parameter estimate for source population effects) between the fully fitted model and the reduced model lacking level-2 variables (stereochemistry) (Singer and Willett, 2003). This statistic provides information about the relative explanatory power of level-1 and level-2 parameters fixed effects (in this case, source population and stereochemistry, respectively).

To investigate whether leaf damage reduced plant fitness, we conducted separate analyses of covariance (ANCOVAs) in Proc Mixed (one for each site in 2009). Plant fitness (bur number \times bur mass) was the response variable, and percentage leaf damage, stereochemistry and source population were predictors. Stereochemistry and per cent leaf damage were treated as fixed effects, and source population was nested within stereochemistry and per cent leaf damage as a random effect. We first parameterized these models to fit separate intercepts and slopes for the different plant chemotypes. We tested whether slopes differed between chemotypes using adjusted means contrasts. As slopes were significantly different from zero but there were no significant differences in slopes between chemotypes, we parameterized a second model to fit separate intercepts but a common slope for the different plant chemotypes. Finally, to

examine whether leaf damage reduced plant fitness under ambient levels of damage, we fit the same models using only plants from the control treatment. In each case, significant negative slopes indicated that damage reduced fitness.

To assess the relationship between stereochemistry and plant fitness in the two 2009 gardens, we conducted nested ANOVA in Proc Mixed. Plant fitness (bur number \times bur mass) 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.

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, an almost tenfold difference ($F_{15,360} = 20.25$, $P < 0.01$, Fig. 2A). Similar patterns were apparent in 2009, with a 5.4-fold difference among populations at KPC ($F_{17,49} = 2.06$, $P = 0.04$, Fig. 2B) and a 12-fold difference at HCC ($F_{17,67} = 1.63$, $P = 0.09$, Fig. 2C).

Is stereochemistry associated with herbivore resistance?

Plants producing *cis*-fused lactones consistently experienced higher levels of herbivory in the field. This pattern held across all site/year combinations: both years at KPC (2008, $F_{1,14} = 27.54$, $P < 0.01$, Pseudo $R_e^2 = 0.45$, Fig. 3A; 2009, $F_{1,16} = 25.8$, $P < 0.01$, Pseudo $R_e^2 = 0.34$, Fig. 3B) and HCC in 2009 ($F_{1,16} = 8.85$, $P < 0.01$, Pseudo $R_e^2 = 0.14$, Fig. 3C). Stereochemistry generally explained a large fraction of the between-source-population variation in herbivore damage (KPC 2008, Pseudo $R_0^2 = 0.67$; KPC 2009, Pseudo $R_0^2 = 1.00$; HCC 2009, Pseudo $R_0^2 = 0.79$).

Does herbivore damage reduce fitness?

Manipulative experiments in 2009 revealed that damage reduced plant fitness, although the strength of the relationship differed across sites (Fig. 4). Planned contrasts revealed no significant differences in slopes between *cis* and *trans* plants at either site, indicating they did not differ in their fitness response to herbivore damage (KPC, $F_{1,16} = 0.25$, $P = 0.62$; HCC, $F_{1,17} = 0.11$, $P = 0.74$). Thus, the relationship between damage and fitness was assessed with the common slopes model at both sites. At KPC, there was a marginally significant trend for fitness to decrease with increases in damage ($t_{1,17} = -2.09$, $P = 0.05$). Alternate analyses using the Kenward–Roger denominator degrees of freedom estimation method showed this relationship was statistically significant when correcting for the low explanatory power of source population in the model ($t_{1,144} = -2.09$, $P = 0.04$). At HCC, there was a

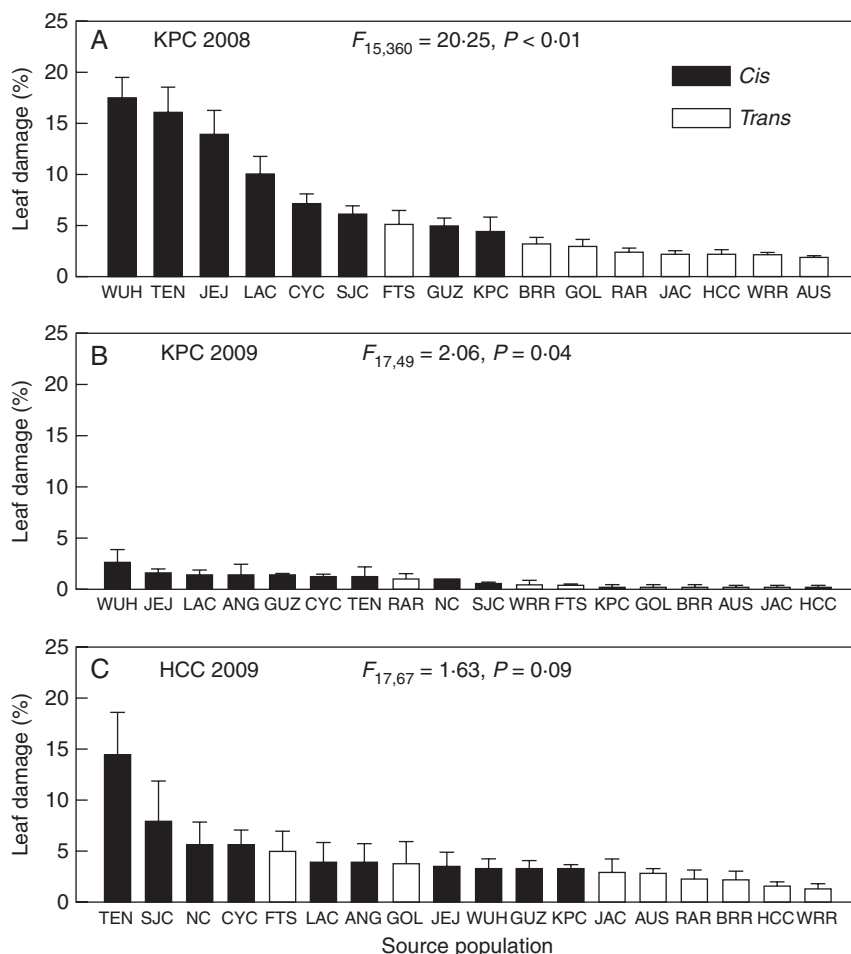


FIG. 2. Levels of herbivore damage experienced by different populations of *Xanthium strumarium* under ambient herbivore conditions in the three experimental gardens (A–C). Data are presented as least squares means (\pm s.e.), with source populations arranged in descending order of percentage leaf damage. Populations with *cis*-fused STLs and populations with *trans*-fused STLs are indicated in the key.

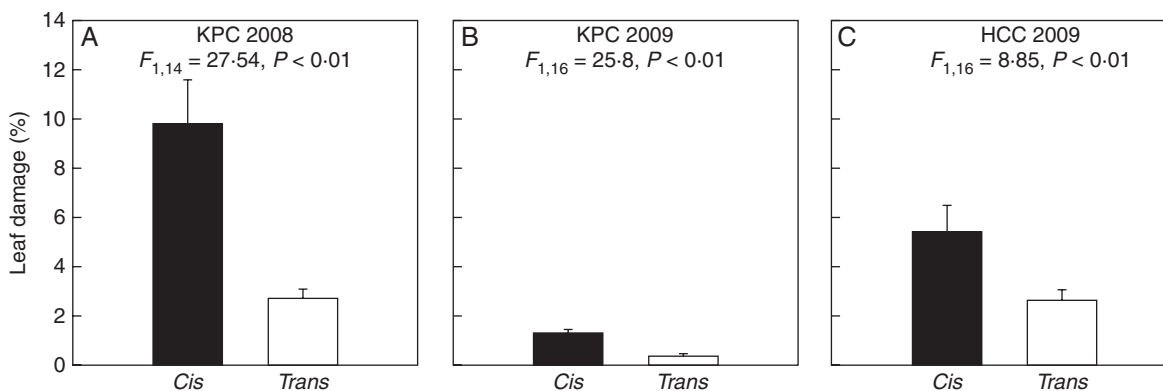


FIG. 3. Stereochemistry predicts the level of leaf damage *X. strumarium* plants experienced under ambient herbivore conditions in the three experimental gardens (A–C). Plants producing *cis*-fused lactones (solid bars) consistently experienced higher levels of herbivore damage than plants producing *trans*-fused STLs (open bars). Data are presented as least squares means (\pm s.e.).

stronger pattern of fitness decreases with increases in damage ($t_{1,18} = -7.44, P < 0.01$). In the control treatment (exposed to ambient levels of herbivore attack), where plants experienced a smaller range of damage, there was no significant relationship

at KPC ($t_{1,16} = -0.47, P = 0.64$), but a significantly negative relationship at HCC ($t_{1,18} = -3.09, P < 0.01$).

As expected from the above results linking stereochemistry to herbivore resistance, stereochemistry was also linked to plant

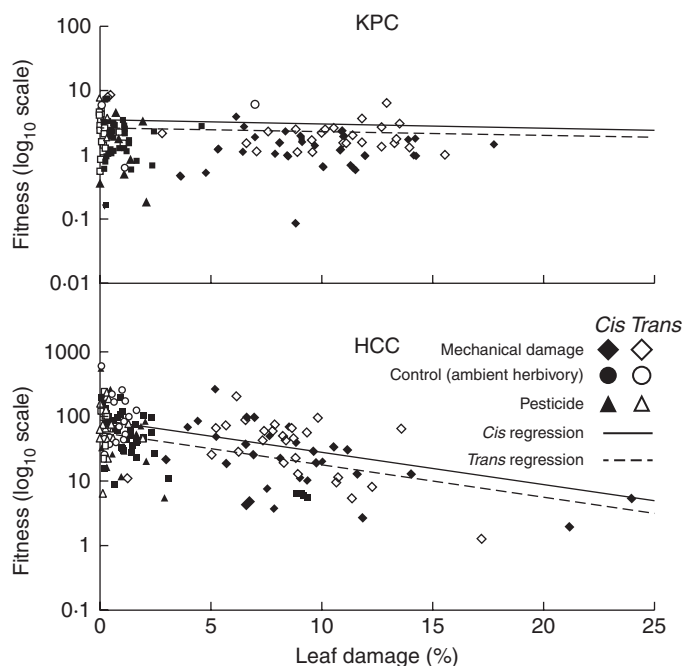


FIG. 4. Relationships between leaf damage and fitness (bur number \times bur mass) for *X. strumarium* in two sites (2009). The statistical significance of regression slopes at each site are as follows: KPC, $t_{1,17} = -2.09$, $P = 0.05$; HCC, $t_{1,18} = -7.44$, $P < 0.01$. Because data were log-transformed in the linear statistical model, regression parameter estimates are back-transformed.

fitness. In 2009, under ambient levels of damage (the control treatment), plants with *trans*-fused STLs had higher fitness than plants with *cis*-fused lactones (KPC, $F_{1,17} = 4.52$, $P = 0.05$; HCC, $F_{1,17} = 5.31$, $P = 0.03$). However, under experimentally reduced levels of herbivory (pesticide treatment), chemotypes did not differ in fitness (KPC, $F_{1,17} = 0.30$, $P = 0.59$; HCC, $F_{1,17} = 3.00$, $P = 0.10$).

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 leaf-chewing insects, consistent with evidence of a heritable component of resistance. Chemotyping separated source populations into two chemotypes based on the lactone ring junction: plants producing *cis*- or *trans*-fused STLs. 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 leaf damage can reduce plant fitness for both chemotypes. 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 STL effects on plant fitness are mediated via herbivores, and furthermore that the costs associated with producing *cis*-fused vs.

trans-fused lactones do not differ substantially in the absence of herbivores.

The mechanistic basis of differential bioactivity between *cis*- and *trans*-fused STLs

Investigations of structure–activity relationships of STLs have found that a number of structural features can influence activity (particularly the presence of the α,β -unsaturated carbonyl group on the lactone ring), yet the influence that stereochemical variation has on STL activities has not been thoroughly examined (Schmidt, 1999, 2006). To date, only a limited number of laboratory studies have demonstrated that *cis*- and *trans*-fused STLs have differential bioactivities; two studies have shown that *trans*-fused STLs confer higher bioactivities (Takeda *et al.*, 2011; Ahern and Whitney, 2013), while one study shows the opposite pattern (Beekman *et al.*, 1997). These studies did not investigate why stereoisomer activities differed. Stereoisomers can have different bioactivities because the three-dimensional shape and flexibility of molecules can influence their intrinsic reactivity with different substrates, as well as dictate whether and how efficiently they can interact with target site(s). However, to understand how stereochemical variation may influence bioactivity, specific molecular targets must first be identified. This may be difficult with STLs, as they exhibit broad-spectrum activity against a variety of molecular targets (Schmidt 1999). As yet, no specific molecular targets have been identified to be associated with STL anti-herbivore activity, although γ -aminobutyric acid (GABA)-type taste receptors may be involved (Mullin *et al.*, 1992; Passreiter and Isman, 1997). Further research is needed to identify specific molecular targets associated with STL anti-herbivore activity before we can understand why *cis*- and *trans*-fused STLs exhibit different bioactivities.

Influence of stereochemical vs. other chemical variation

We have demonstrated strong correlations between STL lactone junction stereochemistry and patterns of leaf chewing damage in the field. These results match those from laboratory feeding assays using isolates of single compounds from *X. strumarium*, indicating that *trans*-fused STLs are more deterrent to chewing insects than *cis*-fused STLs when presented in equal concentrations (Ahern and Whitney, 2013). 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 STLs may benefit not only from a more bioactive stereoisomer, but could conceivably also overall produce more STLs, or produce higher quantities of a more deterrent or toxic form than those produced by plants with *cis*-fused STLs.

Neighbourhood-mediated effects on herbivores

With respect to stereochemistry, *X. strumarium* forms predominantly monomorphic populations in nature, yet plants in our experimental common gardens comprised spatially randomized polymorphic populations. It is well documented that a plant's interaction with herbivores can be influenced by the

composition of the neighbouring plant community (reviewed by Agrawal *et al.*, 2006). For example, a plant can experience either lower or higher levels of damage depending upon the identity and relative palatability of its neighbouring plants, phenomena termed associational resistance and associational susceptibility, respectively (reviewed by Barbosa *et al.*, 2009). As a result, plants growing in monomorphic vs. polymorphic populations may experience different levels of herbivory (Rautio *et al.*, 2012). If herbivores of *X. strumarium* are influenced by neighbourhood-mediated effects, the magnitude of the differences in herbivore damage between *cis* and *trans* plants could be different (either greater or less) in naturally occurring monomorphic populations vs. in the polymorphic populations described herein.

Natural selection on STLs mediated via insect herbivores

We have demonstrated herbivore-mediated natural selection acting on variation in STL chemistry. The criteria necessary to demonstrate selection by a 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, consistent with evidence of a heritable component of resistance. 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. The negative relationship between damage and fitness was found when herbivore attack was experimentally manipulated, and also held within the ambient damage treatment at the site with higher levels of attack (HCC). It was non-significant (but still negative) at KPC, the site with lower attack levels. Together, these three lines of evidence indicate that there is natural selection on STL stereochemistry 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). To the best of our knowledge, this is the first documented example of natural selection on stereochemical variation in defensive chemicals.

Maintenance of *cis* and *trans*-stereoisomers in *X. strumarium*

An important question remaining is: how is the less herbivore-resistant, *cis*-fused STL phenotype being maintained within *X. strumarium*?

One option is that there may be places or times where plants producing *cis*-fused STLs have a selective advantage, which our observations across three gardens have not been sufficient to discover. For instance, *cis*-fused STLs may confer more resistance to particular herbivores than *trans*-fused STLs. One can envisage a scenario where varying assemblages of herbivore species create a geographical mosaic of selection favouring plants with *cis*-fused or *trans*-fused STLs in different environments. This scenario has often been proposed to explain the maintenance of multiple chemotypes in plant species. For

example, variation in the relative abundance of specialist aphid species has been proposed as an explanation for the geographical distribution of different *Arabidopsis thaliana* chemotypes (varying in glucosinolate side chain elongation) in Europe (Züst *et al.*, 2012).

Alternatively, even if plants producing *trans*-fused STLs are always more resistant to herbivores, the *cis*-fused STL form could be maintained if selective agents other than herbivores impose either direct or indirect selection on this stereochemical trait. For example, differences in the direct biosynthetic production costs of *cis*- vs. *trans*-fused STLs could help maintain both forms (Strauss *et al.*, 2002). We have found no evidence suggestive of such differential costs, but if *trans*-fused STLs were more biosynthetically costly to produce, direct selection would favour plants that produce *cis*-fused STLs in environments where herbivores are rare and thus do not reduce plant fitness. In a second scenario, selection on stereochemistry may be imposed by spatially variable agents other than herbivores (e.g. levels of plant–plant competition, nutrient availability, temperature). Evidence from other discrete trait polymorphism systems suggests that selection from agents not directly associated with the putative function of polymorphic traits may contribute to their maintenance (see Whitney and Stanton, 2004). For example, drought and temperature may impose direct selection on different chemotypes of *Thymus vulgaris* (Thompson *et al.*, 2007).

Stereochemistry in plant defence

Our study suggests that stereochemistry can have large effects on herbivory and fitness, indicating that the stereochemistry of plant secondary metabolites may be a generally important but overlooked factor influencing plant–herbivore interactions.

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. In STLs, it is believed that the stereospecific formation of the lactone ring occurs early in the biosynthetic pathway, and subsequent downstream modifications of these initial metabolites lead to the vast diversity of STLs (Fischer *et al.*, 1979; Ikezawa *et al.*, 2011). The results of this study demonstrate that stereochemical differentiation in these precursors can cascade to characterize a major portion of plants' defensive chemical profiles, influencing herbivore resistance and plant fitness. This phenomenon is probably true of other groups of compounds. For example, polymeric condensed tannins are composed of chains of monomers that can show stereochemical variation, e.g. the stereoisomers catechin and epicatechin. Different ratios of catechin and epicatechin (and thus *cis*- vs. *trans*-fused bonds) in condensed tannins influenced snowshoe hare preference for different diets in laboratory feeding assays (Clausen *et al.*, 1990).

As discussed above (see Introduction), stereochemical variation in the STL lactone junction is widespread across taxa. Within the Asteraceae, *trans*-fused forms appear to be more common: a preliminary compilation of records from the literature (J. R. Ahern *et al.*, unpubl. data) has documented *cis*-fused forms of STLs in 69 genera (in 23 tribes) and *trans*-fused forms in 168 genera (in 39 tribes) with co-occurrence of *cis*- and *trans*-fused lactones in approx. 23 % of genera and approx.

26 % of tribes. While this variation could be selectively neutral, its presence and unequal representation across the Asteraceae suggest a potential signal of natural selection. We argue that future phylogenetically informed studies examining stereochemical variation in this diverse class of compounds could not only be informative about the factors driving the evolution of stereochemical variation, but could also provide novel insights into the evolutionary success and diversification of this large and highly successful family of plants.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and provide full details of the chemotyping of the plant material.

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