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Regional variation in composition and antimicrobial activity of US propolis against *Paenibacillus larvae* and *Ascosphaera apis*

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ABSTRACT

Propolis is a substance derived from antimicrobial plant resins that honey bees use in the construction of their nests. Propolis use in the hive is an important component of honey bee social immunity and confers a number of positive physiological benefits to bees. The benefits that bees derive from resins are mostly due to their antimicrobial properties, but it is unknown how the diversity of antimicrobial activities among resins might impact bee health. In our previous work, we found that resins from different North American *Populus* spp. differed in their ability to inhibit *in vitro* growth of the bee bacterial pathogen *Paenibacillus larvae*. The goal of our current work was to characterize the antimicrobial activity of propolis from 12 climatically diverse regions across the US against the bee pathogens *P. larvae* and *Ascosphaera apis* and compare the metabolite profiles among those samples using LC–MS-based metabolomic methods. Samples differed greatly in their ability to inhibit both bacterial and fungal growth *in vitro*, but propolis from Nevada, Texas, and California displayed high activity against both pathogens. Interestingly, propolis from Georgia, New York, Louisiana, and Minnesota were active against *A. apis*, but not very active against *P. larvae*. Metabolomic analysis of regional propolis samples revealed that each sample was compositionally distinct, and LC–FTMS profiles from each sample contained a unique number of shared and exclusive peaks. Propolis from Aspen, CO, Tuscon, AZ, and Raleigh, NC, contained relatively large numbers of exclusive peaks, which may indicate that these samples originated from relatively unique botanical sources. This is the first study to characterize how the diversity of bee preferred resinous plants in the US may affect bee health, and could guide future studies on the therapeutic potential of propolis for bees.

1. Introduction

Social immunity traits are cooperative behaviors among social insects that contribute to disease resistance on the colony level (Cremer et al., 2007; Wilson-Rich et al., 2009; Evans and Spivak, 2010). Resin collection has been demonstrated as a form of social immunity in ants and honey bees and provides important physiological benefits to these species (Chapuist et al., 2007; Simone et al., 2009). These benefits manifest in honey bees, *Apis mellifera*, as increased adult longevity, decreased brood mortality, and the prevention of chronic immune system up-regulation leading to decreased productivity (Evans and Pettis, 2005; Simone et al., 2009; Nicodemo et al., 2013). The benefits of resins to bees make their study practically important for the beekeeping industry.

Evidence also suggests that resins help bees and ants resist infection by specific pathogens. Nest enrichment with resin improved the survival of wood ant larvae challenged with the ant bacterial and fungal pathogens *Pseudomonas fluorescens* and *Metarhizium anisopliae* (Chapuist et al., 2007). Resins in the nest also protect honey bee colonies from the bee fungal pathogen *Ascosphaera apis* (Simone-Finstrom and Spivak, 2013), and colonies respond to *A. apis* challenge by increasing resin foraging (Simone-Finstrom and Spivak, 2013). The effects of resins against bee bacterial pathogens, such as *Paenibacillus larvae*, in whole colonies remain unclear (Lindenfelser, 1968; Antúnez et al., 2008); however, studies have shown that propolis from several regions can inhibit *P. larvae* growth *in vitro* (Lindenfelser, 1967; Bastos et al., 2008). Several compounds have been isolated from bee-deposited resins in Bulgaria with reported *in vitro* activity against *P. larvae,* including benzyl ferulate, pentenyl ferulate,

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pinocembrin, pinobanksin-3-acetate, and a mixture of caffeate esters (Bilikova et al., 2012).

All races of honey bees collect plant resins from their environments and deposit them in their nesting cavities as 'propolis', though some races deposit relatively little compared to others (Seely and Morse, 1976; Crane, 1990; Simone-Finstrom and Spivak, 2010). For clarity, we refer to 'resin' as the material occurring on the plant or on a bee's corbiculae and 'propolis' as the material that has been deposited in the nest and typically mixed with wax or other substances. Propolis is used as a nest building material in natural and manmade bee hives, and feral honey bees use it to coat the entire inside surface of their nesting cavities (Seely and Morse, 1976). In contrast, honey bees generally deposit comparably little propolis inside smooth manmade bee boxes, but instead use propolis to glue down frames and covers. This behavior has likely led to selection against bee stock that deposit large amounts of propolis, as this can make managing the hive difficult for beekeepers (Fearnly, 2001). However, this selection for easy management may have costs to bee health.

Honey bees make propolis from a wide variety of resins depending on the diversity of resinous plants available in a particular environment (Crane, 1990). Like stingless bees, honey bees make choices among resinous plants that co-occur in the same environment by collecting resin from some plants, but not others (Leonhardt and Blüthgen, 2009; Wilson et al., 2013). Plant resins are generally complex mixtures of phenolic and isoprenoid compounds that function in plant defense against pathogens and pests (Langenheim, 2003), though resin composition and antimicrobial activity can be highly variable between plants in different regions or even among species in the same genus (Lindenfelser, 1967; Bastos et al., 2008; Wilson et al., 2013). The functional diversity of plant resins is relatively unknown, but having a diversity of resins available to make propolis from may have important benefits to honey bee health. The availability of diverse resin sources in the environment may provide functional redundancy (reviewed in Rosenfeld, 2002), where one resin can functionally substitute for another in the event of scarcity. Alternatively, propolis with more well-rounded antimicrobial activity may be produced if multiple resin sources are available, where one resin can compensate for a lack of function in another (termed "functional balance" – Drescher et al., 2014). Both functional redundancy and functional balance have been demonstrated for resin collection in stingless bees (Drescher et al., 2014), thus it becomes very important to test propolis activity against multiple pathogens when considering the functional role of propolis in honey bee health.

In our previous work, we found that resins from different North American *Populus* spp. differed in their ability to inhibit *P. larvae* growth *in vitro* (Wilson et al., 2013). Given the diversity of climate and potential botanical sources of resin in the US (Crane, 1990; Wollenweber and Buchmann, 1997), it is probable that bees in some regions will deposit resins with comparatively greater or lesser antimicrobial activities. This antimicrobial diversity could be biologically relevant to bee social immunity, but an evaluation of the magnitude and extent of this diversity is lacking in the US. The goal of our current work was to characterize and compare the metabolite patterns and antimicrobial activity of propolis from across the US using LC–MS-based metabolomic methods and susceptibility assays against the bee pathogens *P. larvae* and *A. apis*. Our ultimate motivation was to better understand how variations in the botanical landscape may affect honey bee health. In addition, these experiments should give insight into the diversity of bee preferred resinous plants in the US and guide future studies focusing on the therapeutic potential of propolis for bees.

2. Materials and methods

2.1. Collection of propolis samples

Propolis samples were collected from 12 locations in the US in 2009–2010 using commercial propolis traps (Mann Lake Ltd., cat. # HD-370) representing distinct botanical regions ranging from the desert southwest, to the cold-temperate north, to the wet southeast (Chaska, MN; Baton Rouge, LA; Ithaca, NY; Jamestown, ND; Lincoln, NE; Raleigh, NC; Wakinsville, GA; Tucson, AZ; Aspen, CO; Vacaville, CA; Beaumont, TX; Fallon, NV). Propolis was trapped from six different colonies per location, except in NV where 10 colonies were trapped. Propolis samples were pooled to average out differences in colony variables (size, health status, etc.) in each apiary. NV and MN propolis were re-sampled from five and one colonies, respectively, in 2012 for further analysis to confirm the reproducibility of antimicrobial activity. NV propolis was re-sampled ~20 miles from the original site and MN propolis was re-sampled ~30 miles from the original site. All samples were stored in sealed glass jars at −20 °C.

2.2. Extraction of propolis samples

Frozen propolis samples were ground to a fine powder in a coffee grinder and 0.1 g of powdered material was extracted with 2 mL of 70% ethanol by vortexing three times for 30 s intervals. To remove any insoluble materials, the resulting extracts were chilled to precipitate extracted wax and centrifuged at 20,000 *g* for 10 min. at 4 °C with the resulting supernatants recovered. Extract concentration was measured by residue weight after solvent evaporation using vacuum centrifugation.

2.3. Chemical analysis

Propolis extracts were standardized to 1.5 mg/mL in 10% acetonitrile in water for analysis by reversed-phase C_{18} liquid chromatography [Thermo-Fisher (San Jose, CA) Acella LC system equipped with a Waters BEH C_{18} 1.0 × 100 mm, 1.8 μm particle size column; flow rate: 0.13 mL/min, column temperature: 35 °C] coupled to Fourier transform mass spectrometry at 15,000 resolution [Thermo-Fisher Orbitrap XL, electro-spray ionization, negative ion mode (LC–FTMS)]. The Genedata Expressionist for Mass Spectrometry software package (http://www.genedata.com/products/expressionist/mass-spectrometry.html) was used to automatically discover peaks in the raw LC–FTMS data in the form of mass/retention time pairs based on Gaussian peak shape, absolute intensity, charge, and isotopic pattern. Multiply charged peaks and singlet peaks without an isotopic distribution were filtered out of the dataset as they likely represented analytical artifacts. Comparative analyses were performed by pair-wise comparisons of the mass/retention time pair lists produced for each of the 12 regional propolis samples.

Identification of pinocembrin, phenylethyl caffeate, and pinobanksin-3-acetate in propolis samples using external standards was performed by re-analysis of propolis extracts using more sensitive LC–FTMS conditions [Thermo-Fisher Dionex UltiMate 3000 LC system equipped with an Agilent (Santa Clara, CA) XDB-C_{18} 2.1 × 100 mm, 1.8 μm particle size column; flow rate: 0.4 mL/min, column temperature: 40 °C, interfaced to a Thermo-Fisher Q-Exactive hybrid quadrupole orbitrap mass spectrometer at 17,500 resolution in negative ion mode]. Standard compounds were purchased from Sigma–Aldrich (St. Loius, MO) (pinocembrin, phenylethyl caffeate) or Angene Chemical (Hong Kong) (pinobanksin-3-acetate).

2.4. Bacterial inhibition assay

Dilutions of 70% ethanol extracts were added to 96-well microplates and dried to a solvent-free residue under a stream of nitrogen gas. When dried in this way, solvent controls did not have an effect on bacterial growth from any lingering ethanol. The *P. larvae* reference strain (NRRL #B-2605, ATCC 9545, LMG 9820 – ERIC type I) (de Gaaf et al., 2006; Genersch et al., 2006) was obtained from the USDA Agricultural Research Service culture collection (http://nrrl.ncaur.usda.gov/). Liquid cultures of *P. larvae* were grown overnight with shaking at 37 °C in brain/heart infusion broth (BHI, Difco) fortified with 1 mg/L thiamine and diluted 1:100 with fresh BHI in each microplate well. Total bacterial growth was measured as the optical density of the well solution at 600 nm (OD_{600}) using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale CA) after 6 h of shaking and incubation at 37 °C, which is midway through the growth phase of *P. larvae* in our conditions. The OD_{600} of each well at 0 h was subtracted as background. Relative bacterial growth for treated wells was calculated as the percent growth of untreated negative controls, with the absolute OD_{600} of untreated controls ~0.13 AU at $t = 0$ and ~0.60 AU at $t = 6$ h. Microplate wells contained 8, 10, 20, 30, 50, 60, 75, 100, 125, or 175 μg/mL of propolis, with 8 replicate wells per treatment and controls. For comparison, the inhibitory activities of compounds previously reported as active against *P. larvae* (pinocembrin, pinobanksin-3-acetate, and phenyl caffeate) (Bilikova et al., 2012) were evaluated as above.

We also characterized the growth inhibition of tylosin (Sigma–Aldrich), an antibiotic approved for use against *P. larvae* infection in the field. The above procedure was used, except dilutions were made in BHI and thus not dried to a residue before the addition of the liquid culture, as tylosin is very water soluble. The concentration range of tylosin used was 0.01–10 μg/mL.

IC_{50} values were determined by growth curve analysis in SigmaPlot 10 (Systat Software Inc, Chicago, IL) by fitting a four-parameter logistic equation, $y = \min + \frac{\max - \min}{1 + \left(\frac{x}{IC_{50}}\right)^{-\text{Hillslope}}}$, to the sigmodial inhibition curves. Statistical significance between IC_{50} values was determined pair-wise between those samples that could be fit. 95% confidence intervals for the differences between IC_{50} values were calculated as $CI = z \pm \left[1.96(\sqrt{x^2 + y^2})\right]$ where x is the standard error of $IC_{50(1)}$, y is the standard error of $IC_{50(2)}$, and z is the difference between $IC_{50(1)}$ and $IC_{50(2)}$. If the confidence interval of the difference between $IC_{50(1)}$ and $IC_{50(2)}$ did not include 0, then the difference between the two IC_{50} values was taken as significant.

2.5. Fungal inhibition assays

A. apis reference strains were obtained from the ARS Entopathogenic Fungal Culture Collection (http://www.ars.usda.gov/is/np/systematics/fungibact.htm) [USDA #7405 (ATCC MYA-4450, mating type+) and USDA #7406 (ATCC MYA-4451, mating type-)]. Fungi were grown and mated on MY-20 media at 31 °C and spores isolated according to standard methods (Jensen et al., 2013). *A. apis* spores were then tested for propolis susceptibility in microplate assays.

96-well microplates were pre-treated with propolis extracts as above with propolis doses of 0.25, 0.5, 2.5, 5, 25, 50, 75, 100, 125, 150, and 175 μg/mL, with three replicate wells per treatment and six replicate wells per control. Each microplate well containing 180 μL of liquid MY-20 media was inoculated with 1.98×10^6 spores in 20 μL of sterile water. Fungal growth was measured relative to untreated controls (as above) after 65 h after shaking and incubation at 31 °C. We observed a long delay (~50 h) in initial *A. apis* growth, but near-maximum growth was achieved by 72 h. Absolute OD_{600} in control cultures was ~0.13 AU at $t = 0$ h and ~0.8 AU to 1 AU at $t = 65$ h. *A. apis* growth curves were analyzed as above, but outliers within treatment and control groups were excluded from growth curve analysis after detection with Grubbs' test at the 99% confidence level (http://graphpad.com/quickcalcs/Grubbs1.cfm).

3. Results

3.1. Diversity in propolis antimicrobial activity against P. larvae

Most propolis samples differentially inhibited *P. larvae* growth in a dose-dependent manner (Fig 1). Statistical comparisons among 95% confidence intervals of differences between IC_{50} values supported four inhibitory groups of samples (Table 1). Propolis samples from NV, TX, and CO were the most inhibitory ($IC_{50} \leqslant 50$ μg/mL, Table 1), while propolis samples from NE, CA, AZ, and ND were slightly less inhibitory ($IC_{50} \leqslant 80$ μg/mL, Table 1). Propolis from NC and GA showed some inhibitory activity, but did not completely inhibit *P. larvae* growth over the experimental concentration range (Fig 1). Propolis samples from MN, LA, and NY did not show any inhibition over our concentration range (Table 1, Fig 1).

Propolis from NV and MN was re-sampled to confirm their anti-*P. larvae* activities, as they represent the most and least inhibitory samples, respectively. The new NV sample, sampled within 20 miles of the original site, showed similar activity to the original

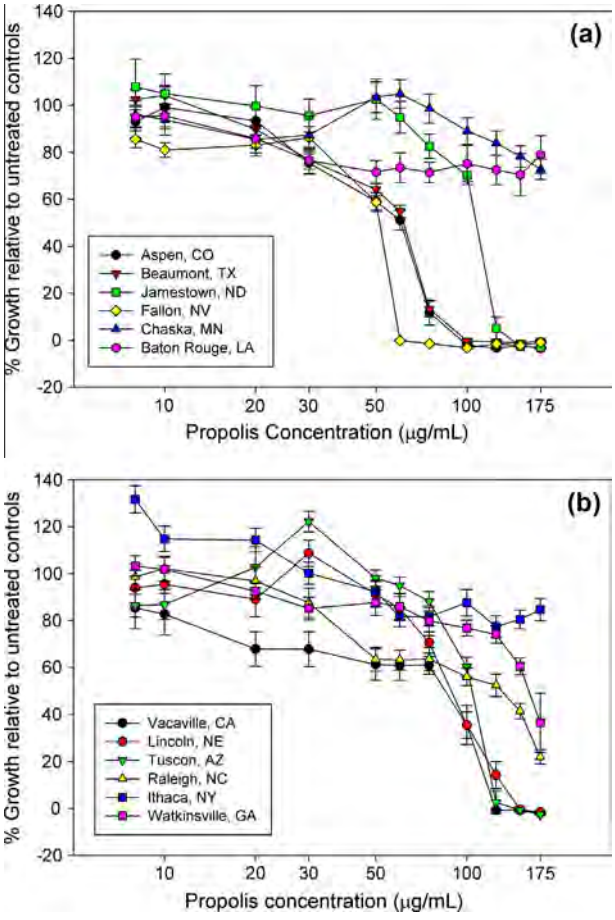

Fig. 1. (a and b) – Inhibition of *P. larvae* growth by propolis extracts. Bacterial growth (y-axis) was measured as a percent optical density (OD_{600}) relative to untreated controls. There were 8 replicate wells per treatment for all propolis samples.

Table 1
IC_{50} values of propolis samples inhibiting *P. larvae* and *A. apis* calculated from dose–response curves. Values were calculated by fitting data points with a four-parameter curve in SigmaPlot 10. R^2 indicates how well the growth data could be fit. Statistical groupings based on non-overlapping 95% confidence intervals for the differences between IC_{50} values are indicated by letters following each IC_{50} value.

Location	*P. larvae*		Location	*A. apis*	
	IC_{50} value (μg/mL)	R^2		IC_{50} value (μg/mL)	R^2
Fallon, NV	41.6 ± 0.5 (a)	0.99	Vacaville, CA	7.4 ± 0.6 (a)	0.97
Beaumont, TX	46.9 ± 2.9 (ab)	0.97	Tucson, AZ	8.3 ± 0.2 (b)	0.99
Aspen, CO	47.1 ± 2.4 (b)	0.98	Fallon, NV	8.6 ± 0.3 (c)	0.99
Lincoln, NE	70.6 ± 2.7 (c)	0.99	Jamestown, ND	10.2 ± 2.4 (abc)	0.99
Vacaville, CA	74.1 ± 4.7 (cd)	0.96	Aspen, CO	10.8 ± 0.9 (d)	0.98
Tucson, AZ	78.4 ± 3.9 (d)	0.96	Beaumont, TX	10[a]	
Jamestown, ND	81.0 ± 2.5 (d)	0.99	Lincoln, NE	10[a]	
Watkinsville, GA	110[a]		Watkinsville, GA	24.2 ± 0.4 (e)	0.99
Raleigh, NC	120[a]		Raleigh, NC	46.4 ± 2.6 (f)	0.99
Chaska, MN	NA		Baton Rouge, LA	46.7 ± 7.0 (f)	0.93
Baton Rouge, LA	NA		Ithaca, NY	48.0 ± 2.7 (g)	0.97
Ithaca, NY	NA		Chaska, MN	81.7 ± 6.8 (h)	0.95
Fallon, NV[b]	52.8 ± 4.8	0.96	Fallon, NV[b]	11.4 ± 2.8	0.97
St. Paul, MN[b]	120[a]		St. Paul, MN[b]	21.9 ± 1.9	0.99

[a] Indicates the value was estimated because the corresponding growth curve could not be fit. IC_{50} values of NA indicate that the sample had low or no activity over the experimental concentration range.
[b] Indicates that these were part of the second trial, as described in the text.

sample (IC_{50} = 52.8 ± 4.8 μg/mL, Table 1); however, the new MN sample, sampled within 40 miles of the original site, showed increased activity (Estimated IC_{50} = 120 μg/mL, Table 1), but did not completely inhibit bacterial growth over the experimental concentration range.

The antibiotic tylosin was much more inhibitory than any of the crude propolis extracts with IC_{50} = 0.255 ± 0.014 μg/mL; however, a direct comparison between tylosin and propolis is not possible because the active compounds in the propolis extracts are unknown and present in unknown concentrations. Pure pinocembrin and phenylethyl caffeate were poorly soluble in BHI broth (50 μg/mL and 30 μg/mL, respectively), but showed some weak inhibitory activity up to their solubility limits. Pinobanksin-3-acetate did not completely inhibit *P. larvae* growth over the concentration range tested, and had an inhibition curve similar to the NC and GA propolis samples. Like those propolis samples, pinobanksin-3-acetate's IC_{50} value was estimated at 120 μg/mL.

3.2. Diversity in propolis activity against A. apis

All propolis samples inhibited *A. apis* growth in a dose-dependent manner (Fig 2), and *A. apis* was much more susceptible to propolis samples than *P. larvae* (Table 1). Statistical comparisons among 95% confidence intervals of differences between IC_{50} values supported eight inhibitory groups (Table 1). Propolis from CA, AZ, NV, ND, and CO were the most inhibitory samples with IC_{50} < 11 μg/mL (Table 1). Propolis from GA was slightly less inhibitory with IC_{50} = 24.2 μg/mL, while propolis from NC, LA, and NY showed IC_{50} < 49 μg/mL (Table 1). Propolis from MN was the least inhibitory with IC_{50} = 81.7 μg/mL (Table 1). Even though propolis from TX and NE completely inhibited fungal growth over the concentration range, the slopes of their inhibition curves were not well defined by the data and could not be fit. IC_{50} values were estimated for these samples at 10 μg/mL (Table 1).

The resampled propolis from NV and MN showed the same trends with *A. apis* as they did with *P. larvae*. Re-sampled NV propolis had similar activity to the original sample with IC_{50} = 11.4 ± 2.8 μg/mL, while re-sampled MN propolis had higher activity than the original sample with IC_{50} = 21.1 ± 1.9 μg/mL.

Although there are no current chemical treatments for *A. apis*, the fungicide benomyl was used as a reference for propolis activity. Unlike tylosin's activity vs. *P. larvae*, the activity of benomyl vs. *A. apis* (IC_{50} = 4.4 ± 1.1 μg/mL) was not substantially greater than the activity of propolis vs. *A. apis*. However, the same caveats discussed previously concerning the comparison of a pure compound to an uncharacterized mixture also apply.

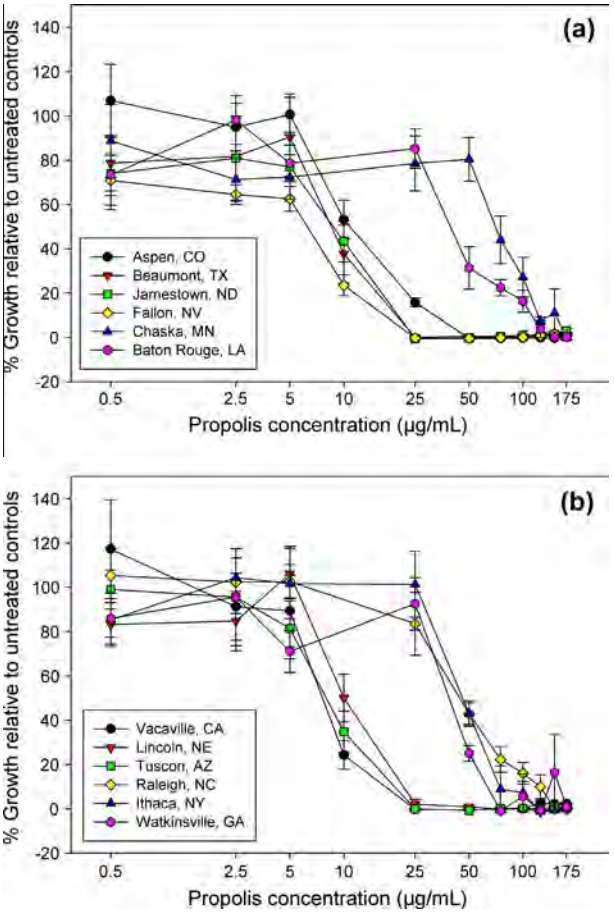

Fig. 2. (a and b) – Inhibition of *A. apis* growth by propolis extracts. Fungal growth (*y*-axis) was measured as a percent optical density (OD_{600}) relative to untreated controls. There were 3 replicate wells per treatment for all propolis samples.

3.3. Metabolomic analysis of propolis samples

Base-peak chromatograms of regional propolis samples are shown in Fig 3. 2188 unique peaks were detected among the 12

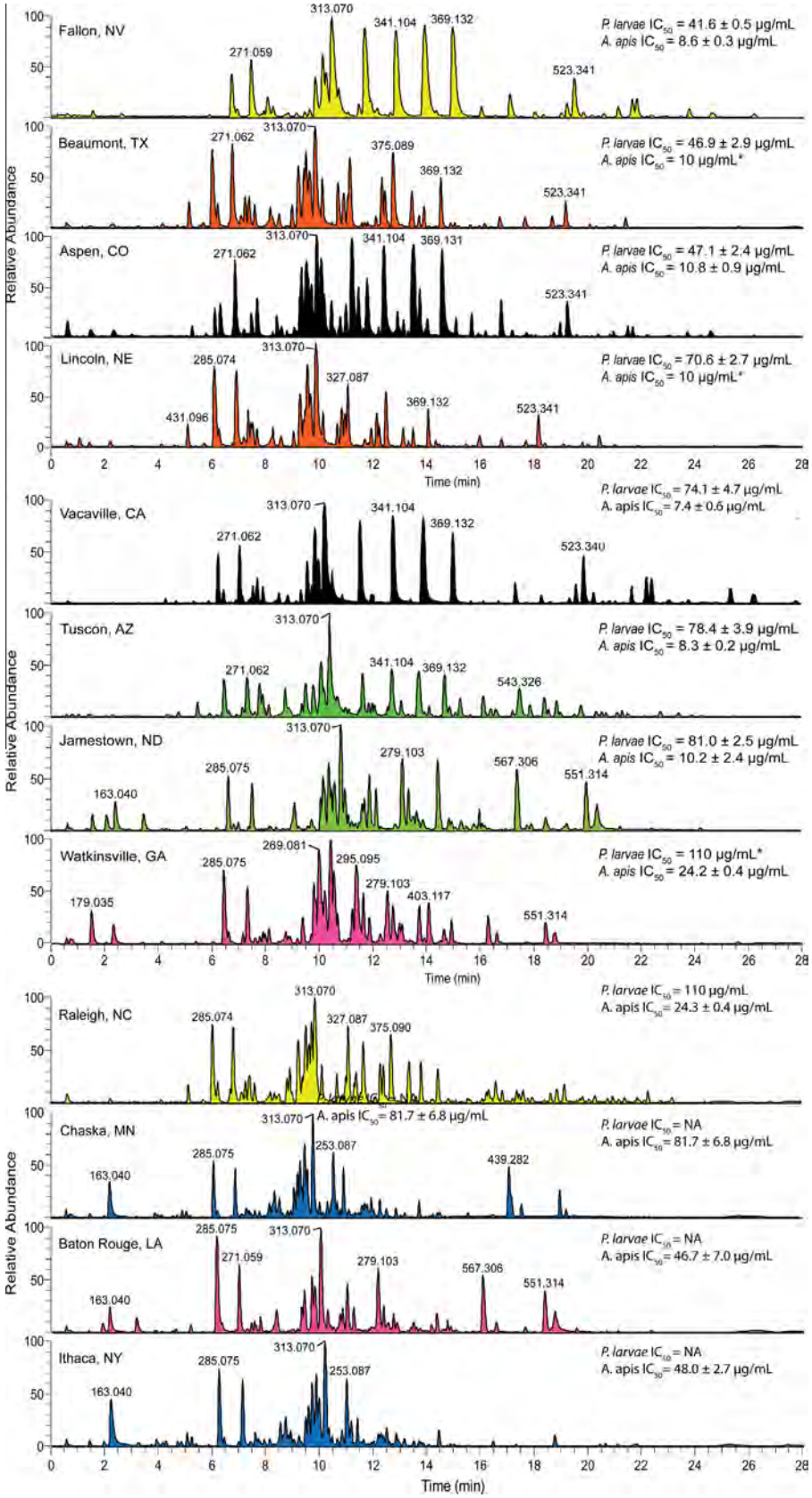

Fig. 3. Base-peak, negative ion LC–FTMS chromatograms of propolis samples. Samples were standardized to 1.5 mg/mL for analysis. Peaks are annotated with the corresponding mass of the base-peak, and colors are coordinated with Figs. 1 and 2. Mass accuracy was 2–5 ppm. IC_{50} values calculated from growth curves (Table 1) are indicated for each sample. IC_{50} values annotated with (*) indicate that the value was estimated because the corresponding growth curve could not be fit. IC_{50} values of NA indicate that the sample had low or no activity over the experimental concentration range. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Compositional comparison of propolis extracts. Pair-wise comparison of peaks found in negative ion LC–FTMS chromatograms of propolis extracts from different regions in the US. The number of pair-wise co-occurring peaks between samples is shown in individual cells. Comparisons between a sample and itself (dark cells) indicate the number of peaks that were exclusive to that sample. The total number of peaks detected in each sample is indicated in the last column. There were 2148 unique peaks in the entire dataset.

	NV	TX	CO	NE	CA	AZ	ND	NC	LA	NY	GA	MN	Peak #
NV	18	371	435	104	291	211	224	304	165	175	212	175	560
TX		3	435	216	317	229	256	364	190	196	239	198	601
CO			93	211	294	224	246	340	185	191	232	197	870
NE				5	177	202	216	196	181	181	208	185	493
CA					9	182	189	265	155	153	179	165	474
AZ						223	229	207	174	180	218	187	948
ND							16	219	190	196	239	198	729
NC								101	170	173	204	180	672
LA									3	173	180	172	651
NY										9	190	181	759
GA											24	192	819
MN												47	756

propolis extracts, but only a subset of the total peaks appeared in any given sample (Table 2). The number of exclusive and pair-wise co-occurring peaks among all propolis extracts are shown in Table 2. Some of the propolis samples with high activity against *P. larvae* and *A. apis* (CO, TX, and NV) shared a relatively large number of peaks, even though these samples came from botanically distinct regions. Some samples had a relatively large number of exclusive peaks (CO, AZ, NC), but there did not appear to be any relationship between the number of exclusive peaks and antimicrobial activity. However, a high number of exclusive peaks might be indicative of unique resin sources only found in these regions.

External standard analysis showed that pinocembrin, pinobanksin-3-acetate, and phenylethyl caffeate were present in all propolis extracts. The relative peak areas of these compounds in each propolis sample did not correlate well ($R^2 < 0.5$) to their corresponding IC_{50} values against *P. larvae*, suggesting that other compounds (either acting individually or synergistically) were primarily responsible for the observed variations in anti-*P. larvae* activity.

4. Discussion

Propolis samples were chemically distinct among different regions (Fig 3, Table 2) and differently inhibited the growth of *P. larvae* and *A. apis* (Figs. 1 and 2, Table 1). Our study showed wide variation in the antimicrobial activity among propolis samples against these bee pathogens, which supports functional differences among propolis produced in different regions of the US Thus, bee-preferred resins available in some areas may be more or less beneficial to bees than others. Furthermore, the observed differences in anti-*P. larvae* activity were not only due to previously reported compounds acting individually, as they were universally present in all propolis samples (active and non-active) and their relative amounts were poorly correlated with anti-*P. larvae* activity. *P. larvae* and *A. apis* are ubiquitous brood pathogens of honey bees in the US, thus propolis activity against them is relevant in all regions represented by our propolis samples. Propolis from NV, TX, CO, CA, and ND seemed functionally balanced (see Drescher et al., 2014) by significantly inhibiting both *P. larvae* and *A. apis* growth. In contrast, propolis from GA, NY, LA, and MN were relatively poor inhibitors of *P. larvae* growth, but relatively good inhibitors of *A. apis* growth (Table 1). This suggests that these landscapes were not functionally balanced in terms of resin availability, thereby containing resins that were poor inhibitors of bacterial growth, but good inhibitors of fungal growth. This differential activity between pathogens illustrates that it is desirable to test multiple pathogens when evaluating the antimicrobial benefits derived from propolis by bees, as it is impossible to predict the overall function of propolis produced in an area without detailed knowledge of its botanical sources and the antimicrobial activities of the resins produced by those species. Our previous work has shown that even resins from closely related plants may fill different antimicrobial niches (Wilson et al., 2013), though we cannot know if the propolis samples in the current study were produced from one or multiple resins without further evaluation. The differential activity of propolis against the two bee pathogens supports that different compounds resulted in propolis antibacterial and antifungal activity, respectively, regardless if they originated from the same or different resins.

It is mostly unknown how propolis affects the 31 diverse organisms that parasitize honey bee colonies (including bacteria, fungi, protozoans, mites, and other insects – Evans and Schwarz, 2011). Evidence does suggests that there are specific functional relationships between resin collection and resistance to fungal and bacterial infections in wood ants (Chapuist et al., 2007) and fungal infections in honey bees (Simone-Finstrom and Spivak, 2013), but we do not know how pathogens are exposed to active resin compounds at the colony level. Since resins are not known to be consumed by ants or bees, resin compounds could interact with pathogens through direct contact, contact with resin compounds adhering to insect cuticles, or volatilization. Both *P. larvae* and *A. apis* are pathogenic through the ingestion of spores (Aronstein and Murray, 2010; Genersch, 2010) and, although our data show that propolis can prevent fungal growth from spores, more research is needed determine the mechanism of propolis inhibition against these pathogens.

Metabolomic methods, including automated peak detection, made more information accessible from spectral data for unique comparisons without requiring the identification of specific compounds, which can be laborious and time consuming. Not only were we able to determine *if* the samples were different from one another, but we were also able to determine overall *how* or *how much* the samples differed from one another in terms of unique or conserved LC–FTMS peaks. In general, US propolis samples appeared to have a number of conserved, high intensity peaks (M–H ions: 271 *m*/*z*, 285 *m*/*z*, and 313 *m*/*z* among others), though each sample also contained a unique pattern of shared and exclusive peaks (Fig 3, Table 2). Some propolis samples with high anti-*P. larvae* and anti-*A. apis* activity shared a relatively large number of peaks (Table 2), but it is impossible to tell from these data if that resulted in their similar antimicrobial activities. Peak differences among samples are likely due to different resinous plants available in the various regions, though little exact information on the

botanical sources of propolis in the US is available (but see Wollenweber and Buchmann, 1997; Crane, 1990; Wilson et al., 2013). The high number of peaks unique to propolis from Tucson, AZ (223 peaks), Raleigh, NC (101 peaks), and Aspen, CO (93 peaks) suggests that chemically divergent resins are available to bees in these regions that are not available in other regions.

The purpose of this study was not to determine if propolis from one region is better than propolis from another, nor was it an attempt to find a specific cure for bee disease (though clearly propolis inhibited bee pathogen growth and some samples were more antimicrobial than others – Table 1). Our study has uncovered potentially biologically relevant variations in both the antimicrobial activities both among regional propolis samples and between pathogens, regional differences in propolis composition, and regions that likely harbor unique, bee-preferred resinous plants. In addition, this work provides a platform from which to rationally select US propolis samples for the isolation of useful antimicrobial compounds. This work furthers our understanding of how the local botanical landscape might influence bee health through variations in propolis function, but perhaps our greatest challenge in the future will be to determine how active resin compounds interact with pathogens in the hive.

5. Conflicts of interest

There are no conflicts of interest to be declared.

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