Distribution Agreement

Brice Lawley April 14, 2015

Within Patch Effects of Pine Biofuel Cultivation on Bee Pollen Carrying Behavior

By

Brice Lawley

Dr. Berry Brosi
Adviser

Department of Environmental Sciences

Dr. Berry Brosi

Adviser

Dr. Christopher Beck

Committee Member

Dr. John Wegner

Committee Member

2015

Within Patch Effects of Pine Biofuel Cultivation on Bee Pollen Carrying Behavior

By

Brice Lawley

Dr. Berry Brosi

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Environmental Sciences

2015

Abstract

Within Patch Effects of Pine Biofuel Cultivation on Bee Pollen Carrying Behavior

By Brice Lawley

Biofuels have become a key source for fuel globally as we move away from fossil fuel use. The US has created mandates which seek to increase fuel produced from biofuels, and the Southeast is a prime source area for biofuel development. Land use change required for increased biofuel cultivation may have negative impacts on biodiversity. This study explores how one critical component of diversity, bees, will be impacted. I observe bees' interactions with flowering plants via changes in pollen carrying behavior within a variety of conditions resulting from traditional vs biofuels-oriented practices concerning pine plantations. I used pollen carrying behavior to understand: 1) If bees were foraging for food successfully and 2) If bees were pollinating plants successfully. I found that open area conditions promoted high foraging success with low pollination success while covered area conditions promoted low foraging success with high pollination success. By implementing a biofuels regime to meet fuel needs, alternating states of poor foraging for bees and poor flowering plant reproduction will result. This will lead to a decline in reproduction for both bees and pollinator-dependent plants.

Within Patch Effects of Pine Biofuel Cultivation on Bee Pollen Carrying Behavior

By

Brice Lawley

Dr. Berry Brosi

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Environmental Sciences

2015

Acknowledgements

My advisor, Dr. Berry Brosi, has been invaluable throughout this process both in terms of practical support and personal guidance. I would like to thank my thesis committee members, Dr. Christopher Beck and Dr. John Wegner, for their much appreciated feedback and their even greater appreciated patience with my work. I thank the senior lab members of the Brosi lab, Carolyn Ayers and Travis Dynes, who both shared advice and support throughout this process. Dr. Karen Bell, a member of the Brosi Lab, was a wonderful teacher in helping me to understand all processes related to DNA barcoding. Emily Dobbs, the lab director of the Brosi Lab, provided a great amount of methodological guidance as well as an immeasurable amount of emotional support. I would especially like to thank one lab member, David Gruenewald, who allowed me to work with him on his project during the summer of 2014 and subsequently inspired this research. I am thankful to another leader of the overarching project, Isabel Gottlieb, for permitting me to join and collect data as well. Thank you, Jamieson Bostch, for working so diligently with me to process all of the data collected. Lastly, I would like to thank the US Department of Agriculture for providing funding for the overarching project of which this study was a piece. I am a thankful recipient of the SIRE Grant and James G. Lester Endowment of Emory University as they made my research possible.

Table of Contents

Introduction

As the global supply of fossil fuels decreases, we must look to alternate fuel sources to meet energy needs. Biofuel, a rapidly renewable source of energy derived from biomass, is one possibility. A shift in favoring biofuels over fossil fuels is occurring at a global scale. Over 40 countries have now introduced measures of some kind to encourage biofuel production (Timilsina 2014). No universal solution exists for what should be grown for biofuel feedstock. Oil palm is one of the most promising crops for biofuel production in Southeast Asia (Fitzherbert et al. 2008), while many South American countries such as Brazil favor sugarcane (Leite et al. 2009). The US has also encouraged a shift to biofuels, yet current biofuels practices prevent us from reaching these production goals. Congressional acts such as the Energy Independence and Security Act (EISA) have made biofuel a necessary part of future fuel production. The Renewable Fuel Standard (RFS), a key provision of EISA, mandates a production rate of 36 billion gallons per year of renewable fuel by 2022, four times that of the mandated 2008 standard (Sissine 2007).

Currently, corn is the most abundant biofuel feedstock for the US, but issues with this source have made this an unrealistic option. Food based or “first generation” biofuels like corn have limited production capacity in the US (Antizar-Ladislao and Turrion-Gomez 2008). Corn-based fuel has been criticized for its energetically inefficient production process (Patzek 2004), possibility of placing food and energy needs at odds (Naik et al. 2010), and production volume that is insufficient to meet energy needs (Antizar-Ladislao and Turrion-Gomez 2008). Much more attractive “second generation,” or “advanced,” biofuels exist. Farmers can either take from residual materials such as timber debris (Davis et al. 2012) or grow material directly for fuel production.

The most promising US region for this, the Southeast, will provide cellulosic ethanol from pine trees. Several advantages exist in this combination of region and feedstock. First, the southeast possesses a longer growing season, allowing for greater productivity when compared to other regions (White et al. 1999). Second, pine already exists as a commercially viable source for cellulosic ethanol due to existing timber reserves and plantations (Davis et al. 2012). Practices are likely to change to meet needs. For example, collection of woody debris, high density planting, and shorter harvest rotations may become favored over current techniques (Davis et al. 2012, Hinchee et al. 2009). Changes to management techniques and expansion of pine cultivation will result in land use change.

Land use change has been identified as having a potentially negative impact on biodiversity (Sala et al. 2000). For oil palms, it was found that biodiversity decreased as area was converted to oil palm plantation (Fitzherbert et al. 2008). It has been previously found that pine plantations had lower biodiversity than more natural habitats (Fletcher et al. 2011). However, it may be the case that such plantations can aid biodiversity in certain contexts (Brockerhoff et al. 2008).

Pollinators are a critical component of biodiversity. Ninety percent of angiosperms require pollinator services in order to reproduce (Burd 1994, Kearns et al. 1998, Ollerton et al. 2011). Land use change has been identified as a potential driver of pollinator loss (Potts et al. 2010), and pollination services have been observed to decline with decreases in natural area (Garibaldi et al. 2011). Bees are of special concern since they provide roughly one third of the world’s agriculturally produced calories and, as a result, also make a disproportionate amount of vitamins and minerals available for human consumption via these pollination services (Klein et al. 2007, Eilers et al 2011).

Many studies have explored the impacts of land use change on bee communities (Verboven et al. 2014, Winfree et al. 2007, Holzschuh et al. 2007). Despite this, little is known about the impacts on functional dimensions, such as interactions between bees and the plants they pollinate. One starting point for understanding changes in plant-pollinator interactions, driven by land use or other anthropogenic changes, is through analysis of patterns of pollen carriage by bees.

This project investigates these interactions through the observing bees’ pollen carriage behavior using two responses. First, to determine bee foraging success, I examined pollen presence/absence on bees. If a large number of bees are found to be carrying pollen in an area, it can be understood that bee food availability is adequate (Kearns and Inouye 1993). Second, to determine plant reproductive success, I examined if bees were successfully pollinating plants via floral fidelity. By looking at which species of pollen are in a pollen load, we can determine how many flower species a bee visited (Kearns and Inouye 1993, Jones 2012). Floral fidelity, the tendency for bees to temporarily specialize to one flower species, is vital for the reproduction of pollination-dependent plants (Brosi and Briggs 2013). Transfer of conspecific (same species) pollen is necessary for reproduction to occur, and transfer of heterospecific (different-species) pollen may reduce reproductive success (Brosi and Briggs 2013, Morales and Traveset 2008, Arceo-Gomez and Ashman 2011). Pollen carriage and pollination success interpreted together can provide insight into pollinator-plant interactions in whatever ecosystem is being examined.

I posed the question: How does pollen carriage behavior differ between traditional and biofuel-oriented longleaf pine cultivation techniques? To answer this question in terms of foraging success, I examined presence to absence pollen ratios for bees by management condition. To answer this question in terms of pollination success, I employed two methods.

First, pollen load composition was assessed via a specialized pollen imaging microscope and software package, the Classifynder. Second, pollen load composition was assessed via DNA barcoding. As previous research suggests that land use change negatively impacts bees in many metrics (e.g. biodiversity) (Sala et al. 2000), I hypothesized that both behaviors observed in this study (proportions of bees carrying pollen and pollination success) would be diminished in conditions which result from biofuels cultivation.

Methods

OVERVIEW

Our team sampled bees from 40 test sites located in historically longleaf pine forested areas of Alabama, Florida, and Georgia from 2013 to 2014 in 8 land use types representing possible biofuels scenarios and current, traditional scenarios (Table 1). The bees were collected using the complementary techniques of aerial netting and pan trapping from late April to early July during each of the two years of the study. We identified all specimens to species and assessed each bee for pollen presence/absence. For 40 of these bees from the Florida sites, I assessed floral fidelity using two methods: 1) a specialized pollen imaging microscope and software package, the Classifynder, and 2) DNA barcoding.

SITE SELECTION

Prior to selecting sites, we identified eight common management styles for examination (Table 1). Clear cut sites as those had all trees recently removed from the site. As debris may be taken for biofuel production (Davis et al. 2012), we contrasted two management conditions of “no residue clear cuts” and “residue clear cuts” with the former having no woody debris left after harvesting and the latter having the post-harvest debris left on-site. We also examined thinning practices as thinned (extracted) material may be taken and used for biofuel production (Hinchee et al. 2009). We contrasted unthinned stands, stands in which no trees were removed during the 12-15 years of growth since planting, with thinned stands, stands in which trees had been selectively removed over the course of their 12-15 years of growth since planting. As biofuel cultivation may favor short-rotation (younger) stands (Hinchee et al. 2009), we also discriminated management style based upon age of the stand being young (8-12 years in age) or old (greater than 15 years in age). Lastly we included two reference conditions for comparison to these variations in management style. We identified longleaf “reference forests” as natural forests of more than 30 years in age with a lower degree of management involving regular controlled burns. Second, the study included corn field sites, representing a major current biofuel group, in which corn was planted at the beginning of the sampling season and permitted to grown and be treated by landowners over the course of the summer.

Table 1. Management condition definitions for sites

Management Condition	Description of Site
Longleaf Pine (Reference)	Longleaf pine trees greater than 30 years in age with low degree of disturbance and management
Clear cut, debris left	All trees removed recently, woody debris remain at site
Clear cut, residues removed	All trees removed recently, no woody debris remain at site
Unthinned, managed	12-15 years of growth for slash or loblolly pine, no trees selectively removed during growth
Thinned, managed	12-15 years of growth for slash or loblolly pine, trees selectively removed during growth
Young, managed	Slash or loblolly pine stand 8-12 years in age
Old, managed	Slash or loblolly stand more than 15 years in age
Corn	Corn crop present and growing throughout collection season

During the summer of 2013, we worked in 12 sites within the state of Georgia. The team collected data in 14 additional sites located in Alabama and another 14 sites located along the Florida-Georgia border both during the summer of 2014. Each region contained at least one site for each of these management conditions.

SAMPLING TRANSECTS

At each site, transects were created by establishing a 200 meter long line with 2 meters of area extended to either side for sampling. Transects were surrounded by at least 1km of the management condition being observed albeit on a patch-level rather than landscape level. Within these transects, we used the complementary techniques of aerial net sampling and pan trap sampling.

AERIAL NET SAMPLING

For aerial net sampling, a collector walked along the 200m transect line of each site for a half hour and, with the aid of an entomological aerial net, caught any bee spotted. The collector paused his timer during the process of handling caught bees so that handling time was not included in the sampling time. Specimens were destructively sampled using ethyl acetate. We conducted aerial net sampling between the hours of 10am and 11am.

PAN TRAP SAMPLING

We also collected bee specimens via pan traps, small plastic disposable bowls painted with UV- reflective paint filled with soapy water (Westphal et al. 2008). Bees are drawn to the UV reflectance, which may mimic the UV reflective nectar guides of flowers (Gronquist et al. 2001), and drown due to the reduced surface tension of the liquid. We evenly distributed a total of 15 traps (5 blue, 5 yellow, and 5 white in color) along each transect line beginning 50m into the transect and alternated the colors of the traps so that no one color was present twice in a row along the transect. We set pan traps at dawn and collected them the morning of the following day.

POLLEN CARRIAGE

After pinning and identifying each bee, I additionally noted if the bee was carrying pollen with the aid of a stereoscope. For female bees, I defined pollen presence as the bee having at least one pollen grain in the appropriate pollen bearing area in regard to the bee's species. Conversely, male bees were counted as having pollen if any was present on their bodies. This distinction was made due to female bees possessing structures which are used to carry pollen back to nests to feed young. For example, bees of the genus *Bombus* usually carry pollen on the corbiculae of their hind legs while bees of the genus *Megachile* usually carry pollen on their abdomens. While male bees do not carry pollen back to nests, they still interact with flowers by feeding upon pollen hence this distinction between male and female pollen "presence."

SUB-SAMPLING FOR FURTHER ANALYSIS

For Classifynder imaging and DNA barcoding, I subsampled the caught bees. Only aerial netted bee specimens were considered as pan traps dissolve and disperse pollen loads of bees within the solution of the trap; contamination could thus have been an issue with pan trapped bees for both the Classifynder and DNA barcoding.

Due to time constraints, only 40 of the 250 qualified bees were analyzed. To choose bees, I eliminated every management condition which did not have at least 4 species with 2 individuals from each species. I then randomly selected four species from each site. In instances where a particular species was represented by more than two individuals, I randomly selected two individuals.

POLLEN COLLECTION FROM BEES

I extracted pollen from each bee within the subsample. I placed each of the 40 bees into a microtube containing a soapy water solution and vortexed the tube repeatedly until no pollen visibly remained on the bee. I removed the bee (to be pinned and identified later) and then centrifuged the microtube containing suspended pollen until the solution was clear once more or a definite pellet had formed at the tip of the tube. I drew off and discarded the supernatant and then stored the microtube containing the pollen pellet at -20^{o}C.

CLASSIFYNDER SLIDE PREPARATION

I prepared slides containing a portion of each extracted pollen pellet. To prepare frozen, pelleted pollen samples for visualization with the Classifynder, I first categorized pollen pellets into small, medium, or large size classes. I added 30µL of ethanol to small pollen pellets; 500µL to medium pollen pellets; and 1000µL to large pollen pellets. I then vortexed each microtube until all of the pollen pellet was suspended in solution, pipetted 10µL of the pollen suspension onto an upside-down coverslip, and allowed all ethanol to evaporate from the cover slip. I then added 10µL of warmed glycerin to a slide and then placed the pollen-containing side of the coverslip onto the glycerin, taking care to prevent bubbles from forming in the glycerin. Once cooled, I sealed the slides by coating the edges of the coverslip with clear nail polish.

CLASSIFYNDER IMAGING AND MORPHOLOGICAL DATA OF POLLEN

After preparing the 40 slides, I imaged each pollen gain on each slide using the Classifynder imaging system, an automated palynology microscope tool which reads slides and captures images of all identified pollen in a given area of the slide (Holt and Bebbington 2014). After the Classifynder finished scanning all of the slides, I went through every pollen image and removed any images which were either unidentifiable or were undesired such as dirt particles, ruptured pollen, and small air bubbles. Using a software feature of the Classifynder, I then took this cleaned image library and exported all associated morphological data, such as area, circularity, and elongation, for each pollen grain image to a spreadsheet.

DNA BARCODING

Using the same 40 pollen samples, I extracted DNA and amplified the *rbcL* gene, one of the subunits of the RuBisCO protein, to then be sent to Beckman-Coulter Genomics for sequencing. First, I centrifuged the samples on a benchtop centrifuge until the supernatant became clear and colorless. I then removed the supernatant. I removed a subsample of each pollen pellet (approximately 10 µL) from which to isolate DNA. I disrupted the pollen exine using a Mini-beadbeater (Biospec; Bartlesville, OK), and then extracted DNA using FastDNA SPIN Kits for Soil (MP Biomedical; Solon, OH). For PCR of *rbcL,* one of the two standard DNA barcoding markers for plants (Hollingsworth et al. 2009), I followed manufacturer's instructions for preparing 16S ribosomal RNA gene amplicons for the Illumina MiSeq system products for sequencing. I followed the manufacturer's instructions for preparing 16S ribosomal RNA gene amplicons for the Illumina MiSeq system (Illumina, San Diego, CA), with the following exceptions: 1) we used forward and reverse primers, *rbcLaF* and *rbcLaR* respectively, for the *rbcLa* gene (Levin et al. 2003); 2) to account for low template DNA concentrations due to small quantity of starting material, we increased the volume of DNA added to each starting reaction to 10.5 µL; 3) due to the longer PCR product expected, we increased extension time to 1 minute during cycles and 10 minutes for the final extension stage; and 4) to account for low template DNA concentration, we increased the number of cycles in the first round of PCR to 35. Using this method rather than standard PCR enables next-generation sequencing to be conducted at a later date if deemed necessary. PCR products were sent to Beckman-Coulter Genomics for Sanger sequencing (using ABI 3730XL DNA Sequencer, Applied Biosystems, Foster City, CA).

DATA ANALYSIS

For pollen presence/absence data, I conducted Generalized Linear Mixed Effects Models with binomial errors using the lme4 package for the *R* statistical computing platform. This test was chosen due to the data lacking true independence (multiple samples were taken from each site). For this test, all other management conditions were compared to the reference forest condition.

For Classifynder-obtained data, I determined load purity per sample and then assessed overall floral fidelity per management condition. First, we exported all Classifynder morphological data to the JMP (2012) version 10.0.0 (SAS Institute Inc, Cary, NC) statistical analysis program. I performed a kmeans cluster analysis in which morphological data were used to group all pollen grains into groups and gain some understanding of how many species could be found across all loads. To minimize faulty over-differentiation in smaller, similar subgroups, I placed all pollen grains from all pollen loads into a single pool for cluster analysis. Given this large sample size as well as preliminary results, I identified kmeans clustering as the best clustering methodology to use as opposed to a variety of possible hierarchical clustering methodologies such as Ward's method. Specifically, we found that hierarchical clustering tended to always return the same number of clusters (usually 20) despite this being clearly inaccurate when compared to direct observation of pollen image sets. I defined "pure" loads as those consisting of 75% or more of one pollen type dominating a load by pollen count. This percentage was obtained after testing several percentage thresholds and comparing them to the pollen loads' image libraries. I concluded that 75% gave the best balance between being strict enough to disallow false cases of "pure loads" yet was also lenient enough to permit some background noise in accounting for the Classifynder's tendency to over-differentiate pollen types. I performed a Fisher's exact test to determine if management condition significantly impacted the probability of a bee carrying of a pure load. I chose this test in part because the data did not meet criteria for the basic chi-squared test.

For DNA barcoding data, I again determined load purity per sample and then assessed overall floral fidelity per management condition. Results from DNA barcoding were analyzed using Geneious (Biomatters, Auckland, New Zealand) software in which de novo assembly was used to manually remove primer sequences. As this software provides a visualization of each forward and reverse sequence, I counted the number of ambiguous nucleotides in each sequence, and calculated them as a percentage of total sequence. I considered samples pure if the percentage of ambiguities for their sequences was less than 1% (Wilson et al. 2010). If there was only a small amount of PCR product the sample wasn't assessed. If there was clearly a second sequence, but the peaks were lower, it was considered mixed, even if there were no ambiguous nucleotides. I performed a BLAST search to the NCBI GenBank database to determine which species was present in a given load. This was limited in that only the sequence with the strongest reading would be identified. After eliminating inconclusive results from the data set, we again ran a Fisher's exact test to determine if management condition significantly impacted the probability of a bee carrying a pure load. I chose this test because the data produced did not meet criteria for a basic chi-square test.

Results

OVERVIEW

We collected pollen data from 3,909 bees provided by the overarching project of which this study was a part (Gruenewald 2014). Of these, 2,363 were carrying pollen and 1,546 were not. We caught the fewest bees in the "corn" management condition ($n = 120$). We caught the greatest number of bees, just over 30% of the entire sample, in the "clear cut, residues removed" condition ($n = 1230$). This stands in contrast to the "clear cut, debris left" management condition with considerably fewer bees caught ($n = 615$). Younger forests (thinned, managed; unthinned, managed; and young, managed) generally had fewer bees when compared to more aged forests (old, managed and longleaf pine conditions).

POLLEN PRESENCE/ABSENCE

Overall, I found that management condition was highly significant in impacting the proportion of bees carrying pollen ($p = 0.000698$). Bees collected from reference forests had the highest proportions of bees carrying pollen (about 74%), and when compared to longleaf pine forests, all other management conditions significantly differed (all p-values were less than 0.05)

(Figure 1, Table 2). Thinned, managed forests had the lowest proportions (about 44%). Clear cut, debris left conditions differed the least from reference forest conditions. Given that thinned, managed forests has the lowest proportion of bees carrying pollen, it is unsurprising that this management condition differed the most from the reference condition. This was followed closely by unthinned, managed conditions.

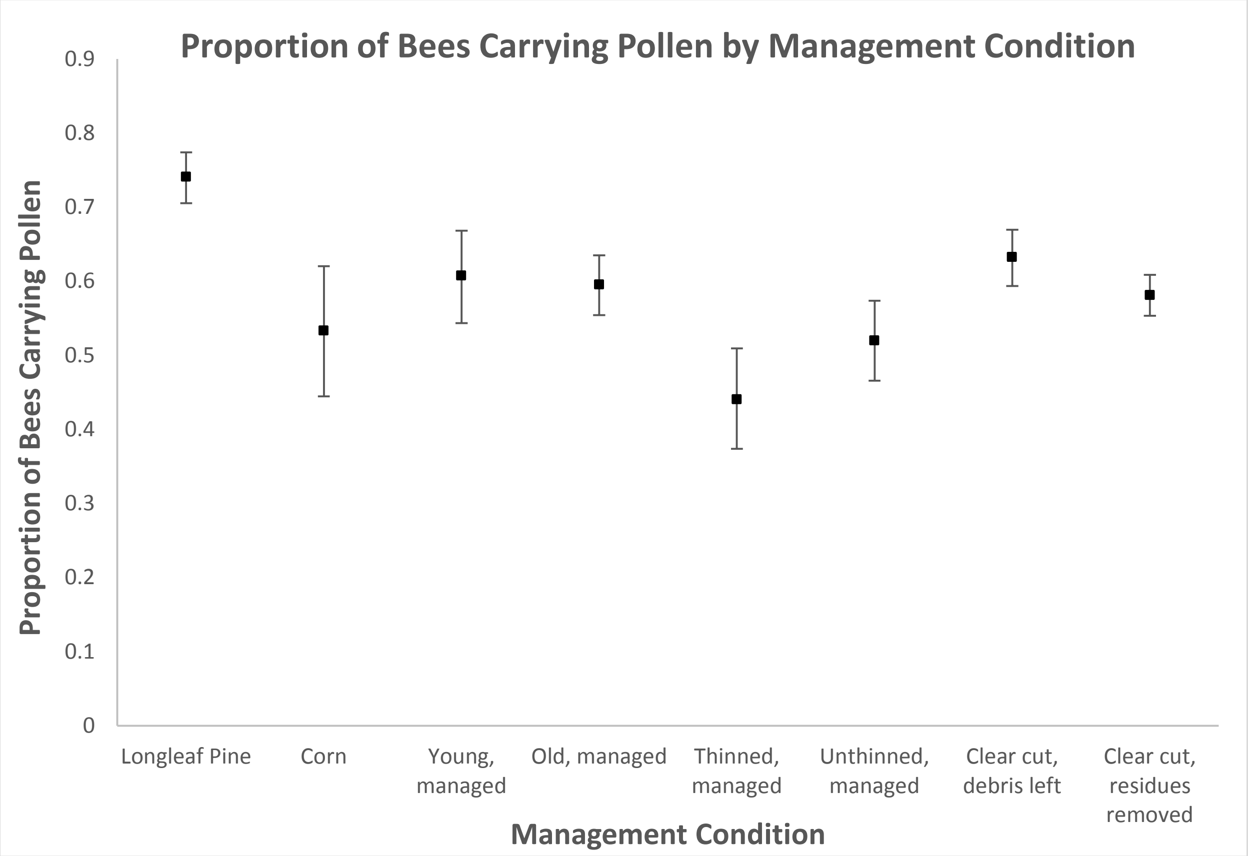

Figure 1: Proportion of bees carrying pollen by management condition. When compared to the reference forest condition, all management conditions were found to significantly differ. All values relating to significance listed in Table 2.

Table 2. All management conditions compared to the reference forest condition using Generalized Linear Mixed Effects Models with binomial errors. When compared to the reference condition, all management types were found to significantly differ ($p < 0.05$).

Management Condition	# Estimate	Standard Error	z-value	p-value
Longleaf Pine (Intercept)	1.12	0.176	6.36	1.99e-10
Corn	-1.08	0.367	-2.93	0.00338
Young, managed	-0.789	0.286	-2.76	0.00577
Old, managed	-0.594	0.240	-2.48	0.0133
Thinned, managed	-1.11	0.331	-3.35	0.000801
Unthinned, managed	-1.08	0.267	-4.05	5.06e-5
Clear cut, debris left	-0.609	0.250	-2.44	0.0148
Clear cut, residues removed	-0.905	0.245	-3.69	0.000226

POLLEN LOAD COMPOSITION - THE CLASSIFYNDER

Across all 40 subsampled bees' pollen loads, 31 different clusters were identified using JMP software's kmeans cluster analyses on the morphological data produced by the Classifynder. I loosely interpreted this to mean that there were 31 different plant species across the 40 pollen loads examined. Using these 31 different clusters, I determined dominant clusters in each load and then inferred the degree of floral fidelity. Defining "pure" loads as those consisting of 75% or higher of one pollen cluster type, I found that one fourth of the loads were relatively pure (suggesting higher floral fidelity for the bees of those loads) (Table 3).The greatest number of purer pollen loads was found in the unthinned, managed condition ($n = 5$) while clear cut, debris left and clear cut, residues removed tied for the least number of purer pollen loads ($n = 0$) despite having some of the highest values both for bees caught and proportion of bees carrying pollen (Table 3). From a Fisher's exact test, I found that management condition significantly impacts the probability that a bee will have a mixed or pure load ($p = 0.0140$).

Table 3. Classifynder Load Purity counts. (*p = 0.0140* from Fisher's exact test).

Management Condition	Count of Impure Loads	Count of Pure Loads	Total Loads
Clearcut, debris left	8	0	8
Clearcut, residues removed	8	0	8
Old, managed	5	3	8
Longleaf Pine	6	2	8
Unthinned, managed	3	5	8

POLLEN LOAD COMPOSITION-DNA BARCODING

Results from DNA barcoding identified up to 14 different species from pollen load compared to the 31 from Classifynder and cluster analysis. Of the 40 pollen load samples sent for sequencing, 32 returned interpretable results. Of those that returned results, 23 (~72%) matched their Classifynder counterparts (Table 5). For clear cut, residues removed management conditions, both methods agreed upon all cases having zero pure pollen loads (Table 5). In the case of clear cut, debris left, however, there were disagreements for 4 (~50% of) cases. In the case of DNA barcoding, both longleaf pine and unthinned management conditions showed much different results when compared to those of the Classifynder; fewer samples were considered pure with reference containing 0 pure loads and unthinned containing 3 pure loads (Table 4). Old managed conditions were found to be the purest condition for DNA barcoding with 6 pure loads compared to the Classifynder showing only 3 pure loads for the this management condition. From a Fisher's exact test, I found that management condition significantly impacts the probability of a bee having a mixed or pure load ($p = 0.0001$). Thus, both Classifynder and DNA barcoding methods agreed.

Table 4. DNA barcoding purity counts ($p = 0.0001$ from Fisher's exact test). Inconclusive loads were those which did not return results for sequencing and were thus left out of analyses.

Management Condition	Count of Inconclusive Loads	Count of Impure Loads	Count of Pure Loads	Total Loads
Clear cut, debris left	0	4	4	8
Clear cut, residues removed	1	7	0	8
Old, managed	2	0	6	8
Longleaf Pine	2	6	0	8
Unthinned, managed	3	2	3	8

Table 5. Comparison of Classifynder and DNA barcoding methods. Loads considered "inconclusive" by the Sanger sequencing technique were not included in this comparison.

Management Condition	Count of inconclusive Loads	Number of Cases Disagreed Upon	Number of Cases Agreed Upon	Total Cases
Clear cut, debris left	0	4	4	8
Clear cut, residues removed	1	0	7	8
Old, managed	2	3	3	8
Longleaf Pine	2	1	5	8
Unthinned, managed	3	1	4	8
Total	8	9	23	40

COMPARISON TO OVERARCHING PROJECT

The overarching project from which this study was created provided further insight into management condition and species composition by providing information regarding flower density and flower species richness (Gruenewald 2014) (D. Gruenewald, personal communication) (Table 6). The overarching project found the greatest flower species richness and relative flower abundance to be in clear cut debris left, longleaf pine forests, and clear cut residues removed conditions.

Table 6. Comparison of this study's findings with the larger, overarching project (Gruenewald 2014) (D. Gruenewald, personal communication).

Management Condition	% Pollen Carriage	% Pure Pollen Loads (Classifynder)	% Pure Pollen Loads (Barcoding)	Relative Bee Density (% of Total Catch)	Bee Species Richness	Relative Flower Density (% of Total Observations)	Flower Species Richness
Longleaf Pine (Reference)	74%	25%	0%	15.9%	41	29.7%	32
Clear cut, debris left	63%	0%	50%	15.7%	54	11.7%	40
Clear cut, residues removed	58%	0%	0%	31.5%	50	33.9%	21
Unthinned, managed	52%	63%	60%	8.4%	36	7.1%	16
Thinned, managed	44%	n/a	n/a	5.2%	38	4.1%	9
Young, managed	61%	n/a	n/a	5.9%	35	4.8%	15
Old, managed	60%	38%	100%	14.4%	51	8.7%	15
Corn	53%	n/a	n/a	3%	21	0%	0

Discussion

The expansion of biofuel production has the potential to result in significant land use change and thus affect community interactions between bees and the plants they pollinate as we transition from traditional pine management techniques to those centered on biofuel cultivation. By comparing various management conditions which could become commonplace under a biofuels regime with those conditions from conventional regimes, I attempted to understand how exactly these changes in bee-plant interactions would be manifested. In this study, we found that management condition had a significant impact on pollen carriage behavior both in terms of the both bee foraging success and successful plant pollination.

In terms of rates of foraging success, longleaf pine forests and clear cut conditions exhibited the highest proportion of bees carrying pollen while younger, denser stands had the lowest proportion of bees carrying pollen. While longleaf pine forests and clear cut sites seem superficially different, similarities in abiotic and biotic factors are present. In terms of abiotic factors, both conditions have a large amount of open space (clear cut sites have no trees and longleaf pine forests are often referred to as pine “savannahs” for their range-like conditions). It is likely that reduced shade in open conditions will promote flower growth as more sunlight is able to reach the ground. Conversely, reduced sunlight in closed, shaded conditions (unthinned managed, thinned managed, and young managed) may contribute to lower flower diversity and relative flower abundance as less sunlight allows for less flower growth. In terms of biotic factors, conditions with open area (longleaf pine, clearcut debris left, and clearcut residues removed) all had higher flower diversity and relative flower abundance (possibly as a result of increased sunlight) (Table 6) (Gruenewald 2014) (D. Gruenewald, personal communication). This high diversity and number of flowers may have also led to these open areas having higher diversity and number of bees given that bees will seek out those food resources. By comparison, biotic conditions may be influence by lack of shade as well. Closed, shaded conditions (unthinned managed, thinned managed, and young managed) had low flower diversity and relative flower abundance which may have then effected low bee diversity and relative bee abundance (Table 6) (Gruenewald 2014) (D. Gruenewald, personal communication). These findings have direct implications for forest management for biofuels. Forest-derived fuels are most efficiently produced in short rotations, and future biofuels scenarios will favor denser stands (Deckmyn et al. 2004, Labrecque and Teodorescu 2005). Thus, pine forests managed for biofuel production could have lower rate of bee foraging success.

I found differences in pollination success among management conditions with lowest floral fidelity being in clear cut and longleaf pine conditions and highest fidelity being in unthinned, managed and old, managed conditions. Floral fidelity, the tendency of bees to specialize on one species of flower for a short time period, is of key concern when understanding how pollinator-plant interactions may change because a particular species of pollen must be transferred to the same species of flower if pollination is to successfully occur (Brosi and Briggs 2013, Morales and Traveset 2008, Arceo-Gomez and Ashman 2011). The cause of floral fidelity differences between management conditions may again be the result of abiotic and biotic differences between open (clear cut and longleaf pine) vs densely treed (unthinned managed, thinned managed, and young managed) conditions. In open conditions, higher sunlight may increase the number of flowers present (and thus floral resources for bees), but may also increase the rate at which bees experience overheating and fatigue. A scenario in which a bee must quickly select pollen from a variety of flowers before overheating may explain why the behavior of floral fidelity is lessened in open conditions. Conversely, well-shaded or vegetation dense conditions may promote floral fidelity in that bees have more time to forage before becoming heat fatigued. This abundance of time may then promote floral fidelity. Biotic factors such as the high floral diversity and relative floral abundance may also effect lessened floral fidelity in open conditions. By offering a much more diverse group of flowers in open conditions, bees may have lessened chance of finding many species of the same flower and thus will resort to mixing pollen loads.

This study highlights the concern that biofuel management conditions may disrupt pollination success by reducing floral fidelity. In comparison to current pine management techniques, clear cut states will likely increase alongside the faster stand rotations expected in biofuel cultivation. More frequent clear cut states mean state which lessen floral fidelity, reducing overall plant reproductive success Another study which assessed floral fidelity in Hawaiian forest restoration plots via bee pollen load composition found that fidelity varied between bee species, but fidelity did not significantly differ between habitat types (Miller et al. 2015). This difference may be due to the sheer difference in types of sites observed, however.

The findings of my project hint at but do not reliably point to specific causes for the changes observed in bee pollen carrying behavior across management conditions, but this could be overcome by additional research. While these patch-level management conditions do provide a general context for these causal components to develop, it would be preferable that direct cause-effect relationships themselves be identified. More needs to be understood about how resulting biotic and abiotic setups within each management condition impact bees’ pollen carrying behavior. Specifically, greater attention should be paid to flowering plant diversity and density. The overarching project also identifies this as a limitation to understanding bee diversity as data collecting regarding flowering plants was relatively limited (Gruenewald 2014). Another specific area which must be addressed is the degree to which open or treed conditions impacts how exposed bees are to abiotic factors (sun, wind, or rain). Do denser tree stands shield from these factors via shade, wind blocking, or foliar absorption? Out of these project findings, three key areas which need further investigation arise. First, to what degree does heat impact foraging success and successful pollination behavior in bees? Second, what specific role does flower diversity and abundance play in foraging success and successful pollination behavior in bees? Third, what impact does tree density have on foraging success and successful pollination behavior in bees?

A large range of studies have been conducted which highlight the negative effects of land use change on bee diversity (as reviewed in Sutherland et al. 2006, Winfree et al. 2009, Kennedy et al. 2013). Despite this attention to bee community metrics, few studies have examined the presence and composition of bee-carried pollen loads. This study shows that examining pollen carriage behavior can provide valuable insights into pollinator-plant interactions. For example, work from the same study which focused on bee communities (Gruenewald 2014) found that clear cut sites had some of the highest diversity and abundance of bees caught. This seemingly good news is put in context by our study’s suggestion that bees from clear cut sites had the lowest incidence of pollen load purity. Thus, despite promoting high bee diversity and abundance, an expansion of biofuel cultivation may lead to lessened flower reproductive success as clear cut states become more frequent.

Despite promising findings, some limitations exist for this study. There are three distinct caveats with the Classifynder. First, the Classifynder is unable to interpret multiple spatial orientations of the same type of pollen. This means that any pollen species with one or more planes of asymmetry could have its collective morphological data show falsely inflated numbers of species for a given pollen load when, in fact, it is the same pollen species imaged from multiple views. A second limitation of this system is its tendency to image non-pollen objects. This issue was remedied somewhat by the process of manually going through each of the pollen images and removing any which were not pollen. Third, the Classifynder had difficulty in accounting for variation between grains of the same pollen type. While a human is able to group varied but similar grains together, the Classifynder will instead place each into its own group. This led to instances in which pure loads were mistakenly over-categorized into a variety of species as damaged pollen grains were assigned to unique clusters perhaps suggesting that Classifynder species estimates were inflated.

For DNA barcoding, there were also challenges. This method is subject to amplification bias in which DNA from a particular species may have simply been amplified more or less effectively due to better matching of universal primers with the gene of interest (Brooks et al. 2015) or higher copy number (Bell et al. *in review*). DNA barocding only identified about 14 unique species in comparison to the Classifynder's 31. One possible explanation is that there are several "background" species which are present in pollen loads, but which never make up the majority of any given pollen load and thus cannot be sequenced. Lastly, low DNA quantity or quality increases the chances of interpreting pollen loads as being impure.

Conclusion

This study found that management conditions significantly alter pollen carrying behavior in bees both in terms of foraging success and successful pollination. Certain management conditions will become more prevalent as mandates shape fuel production practices in Southeastern pine plantations. This study highlights two major points of concern relating to both bees' access to food and plant reproductive success. One condition of biofuels cultivation expected to increase in frequency, clear cut, promotes high bee foraging success yet hinders pollination success. The other condition expected to increase in frequency, the short rotation stand, hinders bee foraging success yet promotes pollination success. As each of these two conditions will repeatedly occur one after the other, concern exists that alternating states of low foraging success and low pollination success will lead to a decline in reproductive success for both bee and flower communities.

References

Antizar-Ladislao, B. and J. L. Turrion-Gomez (2008). "Second-generation biofuels and local bioenergy systems." <u>Biofuels Bioproducts & Biorefining-Biofpr</u> **2**(5): 455-469.

Arceo-Gomez, G. and T. L. Ashman (2011). "Heterospecific pollen deposition: does diversity alter the consequences?" <u>New Phytologist</u> **192**(3): 738-746.

Bell, K. L., K. S. Burgess and B. J. Brosi (in review). DNA Barcoding of Pollen. <u>Plant DNA Barcoding: Methods and Protocols</u>. N. de Vere. New York, Springer.

Brockerhoff, E., H. Jactel, J. Parrotta, C. Quine and J. Sayer (2008). "Plantation forests and biodiversity: oxymoron or opportunity?" <u>Biodiversity and Conservation</u> **17**(5): 925-951.

Brooks, J. P., D. J. Edwards, M. D. Harwich, M. C. Rivera, J. M. Fettweis, M. G. Serrano, R. A. Reris, N. U. Sheth, B. Huang, P. Girerd, V. M. C. a. members), J. F. Strauss, K. K. Jefferson and G. A. Buck (2015). "The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies " <u>BMC Microbiology</u> **15**(66).

Brosi, B. J. and H. M. Briggs (2013). "Single pollinator species losses reduce floral fidelity and plant reproductive function." <u>Proceedings of the National Academy of Sciences</u> **110**(32): 13044-13048.

Burd, M. (1994). "BATEMAN PRINCIPLE AND PLANT REPRODUCTION - THE ROLE OF POLLEN LIMITATION IN FRUIT AND SEED SET." <u>Botanical Review</u> **60**(1): 83-139.

Davis, S. C., M. Dietze, E. DeLucia, C. Field, S. P. Hamburg, S. Loarie, W. Parton, M. Potts, B. Ramage, D. Wang, H. Youngs and S. P. Long (2012). "Harvesting Carbon from Eastern US Forests: Opportunities and Impacts of an Expanding Bioenergy Industry." <u>Forests</u> **3**(2): 370-397.

Deckmyn, G., I. Laureysens, J. Garcia, B. Muys and R. Ceulemans (2004). "Poplar growth and yield in short rotation coppice: model simulations using the process model SECRETS." Biomass and Bioenergy **26**(3): 221-227.

Eilers, E. J., C. Kremen, S. S. Greenleaf, A. K. Garber and A. M. Klein (2011). "Contribution of Pollinator-Mediated Crops to Nutrients in the Human Food Supply." Plos One **6**(6): 6.

Fitzherbert, E. B., M. J. Struebig, A. Morel, F. Danielsen, C. A. Bruhl, P. F. Donald and B. Phalan (2008). "How will oil palm expansion affect biodiversity?" Trends in Ecology & Evolution **23**(10): 538-545.

Fletcher, R. J., B. A. Robertson, J. Evans, P. J. Doran, J. R. R. Alavalapati and D. W. Schemske (2011). "Biodiversity conservation in the era of biofuels: risks and opportunities." Frontiers in Ecology and the Environment **9**(3): 161-168.

Garibaldi, L. A., I. Steffan-Dewenter, C. Kremen, J. M. Morales, R. Bommarco, S. A. Cunningham, L. G. Carvalheiro, N. P. Chacoff, J. H. Dudenhoffer, S. S. Greenleaf, A. Holzschuh, R. Isaacs, K. Krewenka, Y. Mandelik, M. M. Mayfield, L. A. Morandin, S. G. Potts, T. H. Ricketts, H. Szentgyorgyi, B. F. Viana, C. Westphal, R. Winfree and A. M. Klein (2011). "Stability of pollination services decreases with isolation from natural areas despite honey bee visits." Ecology Letters **14**(10): 1062-1072.

Geneious Version 7.1.7. 2014. Biomatters Ltd., Auckland, New Zealand.

Gronquist, M., A. Bezzerides, A. Attygalle, J. Meinwald, M. Eisner and T. Eisner (2001). "Attractive and defensive functions of the ultraviolet pigments of a flower (Hypericum calycinum)." Proceedings of the National Academy of Sciences **98**(24): 13745-13750.

Gruenewald, D. (2014). Bee Community Responses in Pine Systems to Future Biofuel Cultivation in Southeastern US. Master of Science, Emory University.

Hinchee, M., W. Rottmann, L. Mullinax, C. Zhang, S. Chang, M. Cunningham, L. Pearson and N. Nehra (2009). "Short-rotation woody crops for bioenergy and biofuels applications." In Vitro Cellular & Developmental Biology **45**(6): 619-629.

Hollingsworth, P. M., L. L. Forrest, J. L. Spouge, M. Hajibabaei, S. Ratnasingham, M. van der Bank, M. W. Chase, R. S. Cowan, D. L. Erickson, A. J. Fazekas, S. W. Graham, K. E. James, K. J. Kim, W. J. Kress, H. Schneider, J. van AlphenStahl, S. C. H. Barrett, C. van den Berg, D. Bogarin, K. S. Burgess, K. M. Cameron, M. Carine, J. Chacon, A. Clark, J. J. Clarkson, F. Conrad, D. S. Devey, C. S. Ford, T. A. J. Hedderson, M. L. Hollingsworth, B. C. Husband, L. J. Kelly, P. R. Kesanakurti, J. S. Kim, Y. D. Kim, R. Lahaye, H. L. Lee, D. G. Long, S. Madrinan, O. Maurin, I. Meusnier, S. G. Newmaster, C. W. Park, D. M. Percy, G. Petersen, J. E. Richardson, G. A. Salazar, V. Savolainen, O. Seberg, M. J. Wilkinson, D. K. Yi, D. P. Little and C. P. W. Grp (2009). "A DNA barcode for land plants." Proceedings of the National Academy of Sciences of the United States of America **106**(31): 12794-12797.

Holt, K. A. and M. S. Bebbington (2014). "SEPARATING MORPHOLOGICALLY SIMILAR POLLEN TYPES USING BASIC SHAPE FEATURES FROM DIGITAL IMAGES: A PRELIMINARY STUDY." Applications in Plant Sciences **2**(8): 9.

Holzschuh, A., I. Steffan-Dewenter, D. Kleijn and T. Tscharntke (2007). "Diversity of flower-visiting bees in cereal fields: effects of farming system, landscape composition and regional context." Journal of Applied Ecology **44**(1): 41-49.

JMP Version 10.0. 2012. SAS Institute Inc., Cary, NC, USA.

Jones, G. D. (2012). "POLLEN ANALYSES FOR POLLINATION RESEARCH, UNACETOLYZED POLLEN." Journal of Pollination Ecology **9**: 96-107.

Kearns, C. A. and D. Inouye (1993). Techniques for Pollination Biologists. Boulder, University Press of Colorado.

Kearns, C. A., D. W. Inouye and N. M. Waser (1998). "Endangered mutualisms: The conservation of plant-pollinator interactions." Annual Review of Ecology and Systematics **29**: 83-112.

Kennedy, C. M., E. Lonsdorf, M. C. Neel, N. M. Williams, T. H. Ricketts, R. Winfree, R. Bommarco, C. Brittain, A. L. Burley, D. Cariveau, L. G. Carvalheiro, N. P. Chacoff, S. A. Cunningham, B. N. Danforth, J. H. Dudenhoffer, E. Elle, H. R. Gaines, L. A. Garibaldi, C. Gratton, A. Holzschuh, R. Isaacs, S. K. Javorek, S. Jha, A. M. Klein, K. Krewenka, Y. Mandelik, M. M. Mayfield, L. Morandin, L. A. Neame, M. Otieno, M. Park, S. G. Potts, M. Rundlof, A. Saez, I. Steffan-Dewenter, H. Taki, B. F. Viana, C. Westphal, J. K. Wilson, S. S. Greenleaf and C. Kremen (2013). "A global quantitative synthesis of local and landscape effects on wild bee pollinators in agroecosystems." Ecology Letters **16**(5): 584-599.

Klein, A. M., B. E. Vaissiere, J. H. Cane, I. Steffan-Dewenter, S. A. Cunningham, C. Kremen and T. Tscharntke (2007). "Importance of pollinators in changing landscapes for world crops." Proceedings of the Royal Society B-Biological Sciences **274**(1608): 303-313.

Labrecque, M. and T. I. Teodorescu (2005). "Field performance and biomass production of 12 willow and poplar clones in short-rotation coppice in southern Quebec (Canada)." Biomass and Bioenergy **29**(1): 1-9.

Leite, R. C. D., M. Leal, L. A. B. Cortez, W. M. Griffin and M. I. G. Scandiffio (2009). "Can Brazil replace 5% of the 2025 gasoline world demand with ethanol?" Energy **34**(5): 655-661.

Levin, R. A., W. L. Wagner, P. C. Hoch, M. Nepokroeff, J. C. Pires, E. A. Zimmer and K. J. Sytsma (2003). "Family-level relationships of Onagraceae based on chloroplast rbcL and ndhF data." American Journal of Botany **90**(1): 107-115.

Miller, A. E., B. J. Brosi, K. Magnacca, G. C. Daily and L. Pejchar (2015). "Pollen Carried by Native and Nonnative Bees in the Large-scale Reforestation of Pasturelandd in Hawai'i: Implications for Pollination." Pacific Science **69**(1): 67-79.

Morales, C. L. and A. Traveset (2008). "Interspecific pollen transfer: Magnitude, prevalence and consequences for plant fitness." Critical Reviews in Plant Sciences **27**(4): 221-238.

Naik, S. N., V. V. Goud, P. K. Rout and A. K. Dalai (2010). "Production of first and second generation biofuels: A comprehensive review." Renewable & Sustainable Energy Reviews **14**(2): 578-597.

Ollerton, J., R. Winfree and S. Tarrant (2011). "How many flowering plants are pollinated by animals?" Oikos **120**(3): 321-326.

Patzek, T. W. (2004). "Thermodynamics of the corn-ethanol biofuel cycle." Critical Reviews in Plant Sciences **23**(6): 519-567.

Potts, S. G., J. C. Biesmeijer, C. Kremen, P. Neumann, O. Schweiger and W. E. Kunin (2010). "Global pollinator declines: trends, impacts and drivers." Trends in Ecology & Evolution **25**(6): 345-353.

Sala, O. E., F. Stuart Chapin , III, J. J. Armesto, E. Berlow, J. Bloomfield, R. Dirzo, E. Huber-Sanwald, L. F. Huenneke, R. B. Jackson, A. Kinzig, R. Leemans, D. M. Lodge, H. A. Mooney, M. n. Oesterheld, N. L. Poff, M. T. Sykes, B. H. Walker, M. Walker and D. H. Wall (2000). "Global Biodiversity Scenarios for the Year 2100." Science **287**(5459): 1770-1774.

Sissine, F. (2007). Energy Independence and Security Act of 2007: a summary of major provisions. , LIBRARY OF CONGRESS WASHINGTON DC CONGRESSIONAL RESEARCH SERVICE.

Sutherland, W. J., S. Armstrong-Brown, P. R. Armsworth, T. Brereton, J. Brickland, C. D. Campbell, D. E. Chamberlain, A. I. Cooke, N. K. Dulvy, N. R. Dusic, M. Fitton, R. P. Freckleton, H. C. J. Godfray, N. Grout, H. J. Harvey, C. Hedley, J. J. Hopkins, N. B. Kift, J. Kirby, W. E. Kunin, D. W. Macdonald, B. Marker, M. Naura, A. R. Neale, T. Oliver, D. Osborn, A. S. Pullin, M. E. A. Shardlow, D. A. Showler, P. L. Smith, R. J. Smithers, J. L. Solandt, J. Spencer, C. J. Spray, C. D. Thomas, J. Thompson, S. E. Webb, D. W. Yalden and A. R. Watkinson (2006). "The identification of 100 ecological questions of high policy relevance in the UK." Journal of Applied Ecology **43**(4): 617-627.

Timilsina, G. R. (2014). "Biofuels in the long-run global energy supply mix for transportation." Philosophical Transactions of the Royal Society a-Mathematical Physical and Engineering Sciences **372**(2006).

Verboven, H. A. F., R. Uyttenbroeck, R. Brys and M. Hermy (2014). "Different responses of bees and hoverflies to land use in an urban–rural gradient show the importance of the nature of the rural land use." Landscape and Urban Planning **126**(0): 31-41.

Westphal, C., R. Bommarco, G. Carre, E. Lamborn, N. Morison, T. Petanidou, S. G. Potts, S. P. M. Roberts, H. Szentgyorgyi, T. Tscheulin, B. E. Vaissiere, M. Woyciechowski, J. C. Biesmeijer, W. E. Kunin, J. Settele and I. Steffan-Dewenter (2008). "MEASURING BEE DIVERSITY IN DIFFERENT EUROPEAN HABITATS AND BIOGEOGRAPHICAL REGIONS." Ecological Monographs **78**(4): 653-671.

White, M. A., S. W. Running and P. E. Thornton (1999). "The impact of growing-season length variability on carbon assimilation and evapotranspiration over 88 years in the eastern US deciduous forest." International Journal of Biometeorology **42**(3): 139-145.

Wilson, E. E., C. S. Sidhu, K. E. LeVan and D. A. Holway (2010). "Pollen foraging behaviour of solitary Hawaiian bees revealed through molecular pollen analysis." Molecular Ecology **19**(21): 4823-4829.

Winfree, R., R. Aguilar, D. P. Vazquez, G. LeBuhn and M. A. Aizen (2009). "A meta-analysis of bees' responses to anthropogenic disturbance." Ecology **90**(8): 2068-2076.

Winfree, R., T. Griswold and C. Kremen (2007). "Effect of human disturbance on bee communities in a forested ecosystem." Conservation Biology **21**(1): 213-223.