Psyche

Pollinators under Stress: Taxonomy, Physiology, and Behavior of Insect Pollinators

Guest Editors: Zachary Y. Huang and Tugrul Giray

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Editorial Board

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Editorial

Factors Affecting Pollinators and Pollination

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While it has been known for at least a decade that the colony numbers of the managed pollinator, the Western honey bee *Apis mellifera*, was on the decline, pollinator problem was not well publicized until the Colony Collapse Disorder (CCD) further ravaged the honey bee population in the United States [1]. In addition to honey bees, now it is known that bumble bees [2] and other pollinators [3] are also on the decline, perhaps due to combined effects of pesticide use and habitat destruction by *Homo sapiens*. While in the past the main focus has been on the honey bees, we felt that a special issue that includes other pollinators was warranted. Thus was born this special issue, highlighting various important challenges pollinators face.

Stress from Pathogen. The paper by S. L. Bushmann et al. determined if the prevalence of *Nosema bombi* infection was related to history of commercial bumble bee use. Previous studies suggested that bumble bee rearing facilities can spread parasites to local bumble bee populations, but it is not clear whether use of commercial bumble bees in the field may increase infection rate of a pathogen. The study did not find a higher infection rate of *Nosema* in wild bumblebees (*Bombus spp.*) sampled in lowbush blueberry fields with a history of commercial bumblebee use, compared to those without such a history. However, the study shows for sure one species of bumble bee, *B. terricola*, declined in sampled regions. This was most likely due to *Nosema* infection because about half of the bees were infected. However, it could also be due to other factors that first weakened the immune system of the host bees, then causing a higher rate of infection contributing to the population decline. These factors could be due to suboptimal habitats or pesticides used in the crops (blueberry) that end up in the nectar or pollen that these bees foraged upon. Future studies are needed to determine why this native species suffered such a high rate of infection.

Stress from Transportation. There are specific stressors only experienced by managed pollinators. Case in point, each year, over one million managed honey bee colonies are moved across the United States to California for almond pollination. Imagine being transported across three time zones in a highly crowded environment with poor ventilation—it does not sound healthy. Yet, we understand little about how transportation affects honey bee physiology or behavior. K. Ahn et al. conducted an extensive study with three trials in three different states (California, Georgia, and Michigan) to determine the effects of long distance transportation on honey bee physiology. They used a common source of bees, age-marked them by painting, then split them into two colonies, one experiencing transportation and one being stationary. They measured juvenile hormone titers, reasoning that transported bees will experience more stress, higher mortality of older bees, and thus young bees should develop faster to become foragers. Foragers would have higher juvenile hormone titers. Yet, they found no significant differences in juvenile hormone titers between bees reared in the two types of colonies. Protein content in head or thorax and lipid content in abdomen were also largely not different. The only constant measurement that responded to transportation seems to be the size of the food glands, the hypopharyngeal glands. This gland produces the protein portion of royal jelly or worker jelly, which also has lipid component secreted by mandibular glands. This study suggests that bees experiencing transportation have trouble fully developing their food glands, and this might affect their ability to nurse the next generation of workers.

It would be interesting to determine the lasting effects of this transportation stress: do bees recover at all from such an effect? If so, how much time is needed?

Stress from Climate Change. Climate change is a rather large topic currently, but how climate change would affect pollinators remains unknown. D. L. Delgado et al. presented a model study to tackle this question for honey bees. The authors asked two questions for Puerto Rico. First, what is the relationship between honey yield and climate? Second, what is the extent of its spatial variability under current and future scenarios of climate change? The authors developed a number of bioclimatic models that were used in a geographical information system to identify suitable areas for honey production under current and future scenarios of climate change. Models indicated importance of three bioclimatic variables: (1) temperature seasonality, (2) mean temperature of the wettest quarter negatively correlated with honey yields, and (3) precipitation of the wettest month positively correlated with honey yields. In general, the models showed that both honey yields and areas suitable for honey production will decrease under scenarios of climate change. These results illustrate the possible impacts of climate change on honey bees and a method for investigating impact of environmental factors on pollinators.

Plant-Pollinator Interactions. It is known that both biotic and abiotic factors can affect soil quality, which in turn can significantly impact plant growth, productivity, and resistance to pests. Y. J. Cardoza et al., therefore, examined whether soil enhancements of vermicompost (earthworm compost) affected the behavior and physiology of a bumble bee species *Bombus impatiens*. Soil enhancement seems positive for pollinators because it significantly increased the bees' visit durations and reduced time to first discovery of flowers. Bumblebee workers that had fed on flowers from plants grown on enhanced soil also possessed significantly larger and more active ovaries, suggesting a better nutrition of these flowers to bees. Indeed, pollen from these plants had significantly higher protein content, although sugar content from nectar was higher but not significant. It would be interesting to pursue further what nutrients are enriched in these vermicompost added soils.

Plant-pollinator interactions are often considered as tightly coevolved, mutualistic relationships. However, not all visitors may be efficient pollinators. R. W. M. U. M. Wanigasekara and W. A. I. P. Karunaratne studied the bees visiting flowers of the vegetable crop, *Solanum violaceum*, and the efficiency of buzz pollination by bees on fruit and seed production in Sri Lanka. Four buzzing bee species: *Hoplonomia westwoodi*, *Amegilla comberi*, *Patellapis kaluterae*, and *Xylocopa tenuiscapa*, and 3 nonbuzzing species: *Apis dorsata*, *Trigona iridipennis*, and *Ceratina hieroglyphica* visited the flowers of *S. violaceum*. Buzzing bees were the first to visit *Solanum* flowers and were followed by nonbuzzing bees. Handling time of *H. westwoodi* and *P. kaluterae* varied with the availability of pollen in anthers that deplete with the age of flower and stayed longer at new flowers than at old flowers. Handling time of the larger buzzing bee, *H. westwoodi*, was higher than that of the smaller *P. kaluterae*. The fruit set, seed set, and seed germination ability in flowers visited by buzzing bees were significantly higher than those of the flowers bagged to exclude pollinators.

The work on potential pollinators or floral visitors to Brazil nut in the central Amazon rainforest could be a classic because of careful observation and description first being reported in this issue (M. C. Cavalcante et al.). This study is similar to the study by Wanigasekara and Karunaratne which studied bee visitors of one plant species, *Solanum*, but was carried out with cultivated Brazil nut trees (*Bertholletia excelsa* Bonpl., Lecythidaceae) in the Central Amazon rainforest, Brazil. The study aimed to determine pollination requirements and floral visitors of Brazil nut trees and to investigate foraging behavior of these visitors in commercial plantations. Results showed that *B. excelsa* was predominantly allogamous but capable of setting fruits by geitonogamy. Nineteen bee species, belonging to two families, visited and collected nectar and/or pollen throughout the day. Individuals from 16 of 19 bee species succeeded entering the flower and potentially acted as pollinators. However, after considering abundance, flower frequency, and foraging behavior of floral visitors, it was concluded that only two species, *Eulaema mocsaryi* and *Xylocopa frontalis*, could be, considered relevant potential pollinators.

Invasive Species. Invasive species also take part in plant-pollinator interactions. The "tour-de-force" study of pollinators of invasive and native species of *Potentilla* by J. McIver and K. Erickson demonstrates how invasive plants and invasive pollinators could augment the risk to native species. In other words, interactions of invasive species in a region should be considered and not just each invasive species on its own accord! The authors studied four sites in northeastern Oregon, USA in a relatively long-term study (2002 to 2004), and found that invasive plant sulfur cinquefoil and its native congener slender cinquefoil attracted over 100 insect species in 4 orders. Interestingly, compared to the native, nearly twice as many seeds germinated for sulfur cinquefoil, with seeds germinating over a longer period of time. The greater frequency of nonnative pollinators such as honey bees was observed on the invasive *Potentilla* species.

As the subtitle of this special issue indicates, by its nature, studying pollinators requires combining many different fields of expertise such as taxonomy, physiology, behavior, and others. We would like to thank all contributors, many reviewers from diverse fields, and all authors who submitted their work for this issue. Pollination has a great impact on life on earth, and we do hope that this special issue would be a beginning to combine basic and applied studies to expand our knowledge on pollinator biology.

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Tugrul Giray

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Research Article

Forecasting the Influence of Climate Change on Agroecosystem Services: Potential Impacts on Honey Yields in a Small-Island Developing State

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Global change poses numerous challenges to developing nations and small-island developing states (SIDSs). Among these are the effects of climate change on honeybees' provisioning services including honey production. Here we ask two questions. First, what is the relationship between honey yield and climate in a tropical environment? Second, how does yield vary spatially under current climate and future scenarios of climate change? Focusing on the island of Puerto Rico, we developed an ensemble of bioclimatic models that were used in a geographical information system to identify suitable areas for honey production under current and future scenarios of climate change. A comparison between contemporary (1998–2005) and historical (1910–1974) honey yield data revealed a reduction in average yield, including variability, over time, with current yields averaging 5.3 L/colony. Three bioclimatic variables were retained by at least three models: temperature seasonality and mean temperature of the wettest quarter were negatively correlated with honey yields whereas precipitation of the wettest month was positively correlated. The four models varied in terms of their predictions but showed that both honey yields and areas suitable for honey production will decrease under scenarios of climate change. These results illustrate the possible impacts of climate change on honey and ultimately honeybees.

1. Introduction

Honeybees (*Apis mellifera*) and their resource base have been managed to enhance supporting and provisioning services to human kind since ancient times [1–3]. In recent years much emphasis has been placed on the decline of pollination services provided by honeybees both in natural and managed ecosystems in response to multiple drivers of change [4]. This is because pollination directly impacts the functioning of ecosystems and ultimately local and regional economies. Surprisingly, little attention has been paid to potential changes in the delivery of provisioning services such as honey and beeswax production [5, 6]. Understanding these changes is important because beekeeping is promoted as a tool for rural development and conservation in developing nations in the tropics or regions therein (e.g., [7, 8]; http://www.beesfordevelopment.org/).

The importance of honeybees in the delivery of provisioning services is reflected by the widespread introduction of beekeeping practices by Europeans to their colonies in the early 1600s [2]. In the Caribbean, beekeeping was aimed at the production of honey and beeswax sometimes to supply local, and at other times, regional, and international markets [9–11]. Yet the production of honey and beeswax seems to have varied greatly across the islands and within them as illustrated by the island of Puerto Rico where a "boom and bust" cycle occurred in tandem with a decline in honey yields (Figure 1(a); supplementary material available online at doi: 10.1155/2012/951215). Such cycles reflect the often complex dynamics of markets as influenced not only by the social, political, and technological vagaries of a region, but also the interactions between honeybees, their resource base, and environmental factors including meteorological events.

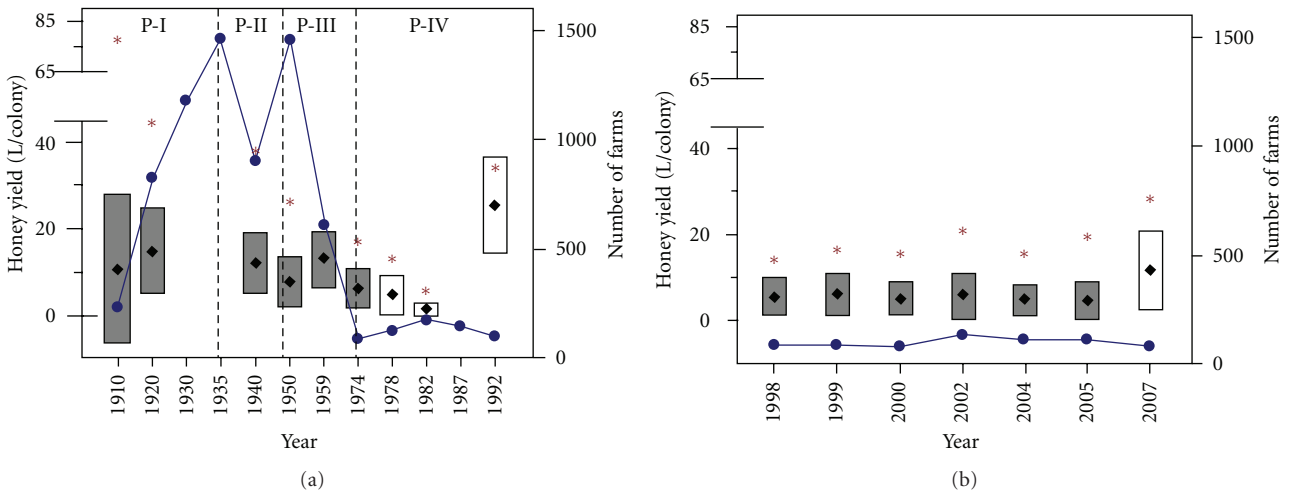

Figure 1: (a) Historical (1910–1974) and (b) contemporary (1998–2005) honey yields for the island of Puerto Rico illustrating the "boom and bust" cycle of honey production. Based on historical accounts, it is possible to distinguish four periods in this cycle and the readers are referred to the supplementary material for details. Average (black diamond) and maximums (asterisks) honey yields, including standard deviations (boxes). In years for which honey yield data is sparse (≤8 municipalities) the standard deviation boxes are in white, otherwise in gray. The closed circles joined by a line depict the number of farms reporting honeybee colonies. Historical data were compiled from historical agricultural census data (supplementary material).

Among the environmental factors that may impact the delivery of provisioning services by honeybees is climate change as the observed variation in honeybee abundance and honey yields along climatic gradients suggests [1, 12, 13]. At low latitudes, honeybees remain active throughout the year whereas at high latitudes they pass through a period of complete inactivity [1, 5, 14, 15]. Likewise within the tropics, the activity of honeybees decreases with increasing elevation [15]. Climate directly influences honeybee behavior given the strong dependency of bee foraging activity and flight on temperature, solar radiation, and wind at a variety of time scales [16, 17]. Climate can indirectly influence honeybees through its effects on their resource base, including flowering plants, pathogens, and predators [6, 18, 19]. Temperature and to a lesser degree precipitation seem to exert a primary control on honeybee activity, yet the extent to which climate change will impact honey yields is poorly understood.

This lack of understanding of the effects of climate change on honey yields, and more broadly speaking the delivery of provisioning services, is prevalent at *regional* to *local scales* particularly in developing regions and small-island developing states (SIDSs) [20, 21]. First, there are large uncertainties regarding the effects of climate change scenarios at increasingly smaller scales due to the coarseness of climate change models and scarcity of climate data [22]. Second, ecosystem services are delivered at local scales but are influenced by processes operating at multiple scales [23]. Lastly, there is an uneven capacity among regions and nations to develop research and technology that could help cope adaptively with global change [24]. Developing regions and SIDS are a point in case. These regions not only face the greatest uncertainties [25–27] but also the greatest vulnerabilities [28] regarding crop production and trade under scenarios of global change. The general consensus is that the productivity of agroecosystems, in particular those delivering the major world food crops, will decrease towards the tropics and subtropics [29]. Therefore, it remains to be seen what happens with the vast majority of crops that are produced, consumed, and traded within regional and local markets, and at the same time are delivered by agroecosystems contributing additional ecosystem services. One such agroecosystem is honeybees and their resource base. Here we ask how honey yields will be impacted by climate change in the island of Puerto Rico, one SIDS within the Caribbean.

We develop four models describing the relationship between honey yields and climate taking advantage of available contemporary (1998–2005) honey yield data for the island of Puerto Rico. Then we analyze these models spatially to identify the areas suitable for honey production under current conditions and future scenarios of climate change. Our approach is based on the development of an ensemble of bioclimatic forecasting models in which the combination of multiple forecasts increases the robustness of the predictions [30]. We reasoned that this approach was necessary given that several regionalized climate change models for the Caribbean show conflicting scenarios for the island of Puerto Rico [31–34]. Therefore, any attempt to evaluate the effects of climate change on the delivery of honey, and more broadly speaking ecosystem services in this region, needs to account for these diverse scenarios.

2. Methods

2.1. Study Site. Puerto Rico with its 8,740 km^2 is the smallest island of the Greater Antilles in the Caribbean (Figure 2). The island has a diverse set of bioclimatic conditions due, in part, to its complex topography and wide elevation range

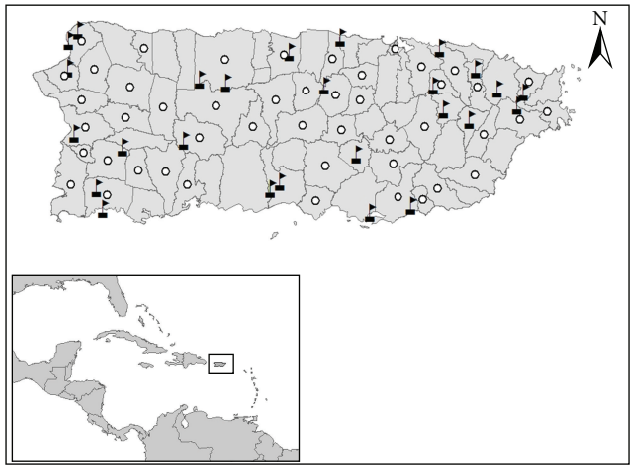

Figure 2: Map of Puerto Rico showing its position within the Caribbean basin, the centroids of the municipalities with contemporary (1998–2005) honey yield data (circles) and the location of the weather stations (flags) with existing precipitation and maximum and minimum temperatures for the same period.

(0–1,338 m; [35–39]). This diversity in combination with available data on contemporary beekeeping activity makes the island ideal for examining the relationship between honey yields and climate variability.

The surplus of honey produced per colony or beehive, that is, honey yields, integrates management and environmental factors that directly and indirectly influence honeybee activity [40]. Modeling honey yields as a function of climate involved two steps: the compilation and creation of honey yield and climate spatial databases; the modeling of honey yields as a function of bioclimatic variables. The latter was generated in BIOCLIM, a predictive system developed for the purpose of modeling the distribution of animals and plants, including agriculturally important crops [41, 42].

2.2. Spatial Databases. We used unpublished beekeeping census data for the period 1998–2005 to calculate honey yields at the municipality level (Figure 2). These data collected by the Department of Agriculture of Puerto Rico (DAPR), with some exceptions, mirror the historical agricultural census data (Figure 1(a)). The DAPR collects statistical data through surveys conducted every two years among beekeepers on the location of the beehives or bee colonies, the number of bee colonies, the total volume of honey produced, the amount of honey sold, and the income generated from the sale during the previous and the current year (A. M. Cruz pers. comm.). These unpublished census data exclude information on beeswax production. To maintain data confidentiality, DAPR does not disclose the exact location of bee colonies; instead they report the corresponding municipality. This may reduce the spatial resolution of the data and limit the possibility to verify unusual data points with the beekeepers. Nevertheless, this dataset provides valuable temporal and spatial information about honey yields on a regional scale (Figure 2).

For each beekeeper, honey yields were calculated dividing the total volume of honey produced by the number of colonies that they reported. Then for each municipality we averaged honey yields across beekeepers to obtain yearly (1998–2005 period; the years 2001 and 2003 were excluded because of incomplete or missing data) and overall (6 years) honey yields. The mean number of beekeepers per municipality ranged between 2.4 and 2.8 and the average number of municipalities with honey yield data was 30 ± 7 out of a total of 78.

We compiled monthly total precipitation and monthly average maximum and minimum temperatures from those weather stations whose records matched the time period covered by the DAPR's honey yield datasets. A search of the National Oceanic and Atmospheric Administration's (NOAA) cooperative weather stations (http://www.ncdc.noaa.gov/oa/ncdc.html), the Luquillo Long-Term Ecological Research Site (http://luq.lternet.edu/), and the Atmos Carib Research Center at the University of Puerto Rico-Mayaguez Campus (http://atmos.uprm.edu/) databases yielded 126 weather stations of which 21 met closely our criteria (Figure 2). Five of these 21 stations had continuous monthly data for the 6 years covered by this study (72 months). The remaining stations had ≤19 months with missing data and 2 among these had a full year of missing data (2003 and 2005 for Mayagüez city and Pico del Este stations). We completed the monthly missing data as follows: averaging monthly values across two adjacent years to complete months without data when daily data did not exist (Case 1), averaging daily values for a given month when incomplete daily data existed (Case 2), and predicting missing monthly data using linear regression models that related climate data from nearby stations (Case 3) in the case of Mayagüez city and Pico del Este stations.

The honey yield and climatic data were added as attributes of point features in a GIS (State Plane Puerto Rico and Virgin Island FIPS 5200, NAD 83) to create interpolated surfaces with a 450 m resolution that were needed as input data in our spatially explicit modeling approach. In ArcGIS 9.2, we used the inverse distance-weighted (IDW) method with its default values (power = 2 and maximum number of neighbors = 15) to interpolate the yearly and overall honey yields added to the centroids of each municipality, the yearly and overall monthly total precipitation, and averaged monthly maximum and minimum temperatures added to each weather station (Figure 2).

We chose the IDW method over others (splice, kriging, natural neighbors) because it produced the interpolated surfaces that most resembled the actual climatic distribution in the island. IDW is a local, deterministic interpolation method that estimates unknown point values based on known neighboring sample points, whose influence decreases with distance according to a negative power function [43]. The error of the interpolated surfaces can vary with P and neighborhood characteristics and also with the variable under consideration [44–46]. We used a cross-validation

Table 1: Bioclimatic variables calculated by BIOCLIM software and used in the stepwise multiple regressions to produce the four honey yield predictive models.

BIOCLIM variable	Description	Type
Bio 1	Annual mean temperature (°C)	Annual trend
Bio 2[1,2]	Mean monthly range (°C)	Seasonality
Bio 3[1,2]	Isothermality (Bio 2/Bio 7)	Seasonality
Bio 4[1,2,3,4]	Temperature seasonality (CV × 100)	Seasonality
Bio 5[1,2,3,4]	Maximum temperature of warmest month (°C)	Extreme conditions
Bio 6[1,2,3,4]	Minimum temperature of coldest month (°C)	Extreme conditions
Bio 7	Temperature annual range (Bio5-Bio6) (°C)	Seasonality
Bio 8[1,2,3,4]	Mean temperature of wettest quarter (°C)	Extreme conditions
Bio 9[1,2,4]	Mean temperature of driest quarter (°C)	Extreme conditions
Bio 10	Mean temperature of warmest quarter (°C)	Extreme conditions
Bio 11	Mean temperature of coldest quarter (°C)	Extreme conditions
Bio 12	Annual precipitation (mm)	Annual trend
Bio 13[1,2,3,4]	Precipitation of wettest month (mm)	Extreme conditions
Bio 14[1,2,4]	Precipitation of driest month (mm)	Extreme conditions
Bio 15[1,2,3,4]	Precipitation seasonality (CV × 100)	Seasonality
Bio 16[4]	Precipitation of wettest quarter (mm)	Extreme conditions
Bio 17[1,2,3,4]	Precipitation of driest quarter (mm)	Extreme conditions
Bio 18[1,2,3,4]	Precipitation of warmest quarter (mm)	Extreme conditions
Bio 19	Precipitation of coldest quarter (mm)	Extreme conditions

A quarter represents a 3-month period. The superscripts indicate the variables that were preselected by PCA and VIF analyses based on multicollinearity to enter the models (see text).

procedure on some selected variables and varied P (1, 2, 3) and the maximum number of neighbors (10, 15) to examine their impact on the root mean square errors (RMSEs). We found that RMSE varied minimally, and therefore kept the interpolated surfaces created with the IDW default values.

These interpolated climatic maps were used together with a digital elevation model (DEM; seamless.usgs.gov) in DIVA's BIOCLIM module to generate yearly and overall bioclimatic variables (Table 1; DIVA version 5.4.0.1; http://www.diva-gis.org/).

2.3. Modeling Contemporary Honey Yields. We developed an ensemble of four models that altogether provide a robust representation of current and future predicted honey yields [30]. The models reflected different ways of aggregating the data (yearly versus overall averages) and handling regions (all versus subset of municipalities) for which the bioclimatic data may not fully represent existing conditions. Model 1 included yearly data for all municipalities for which honeybee data was available. In model 2, we included yearly data for all but four municipalities located in eastern Puerto Rico, namely Ceiba, Naguabo, Luquillo, and Rio Grande. Although these municipalities encompass coastal areas that are characteristically dry, all operating weather stations in the area are clustered in the Luquillo Mountains that are characteristically wet. In Model 3, we averaged yearly values for all the municipalities for which we had honey yield data. Finally, in Model 4 we used the average yearly values of all variables as in Model 3 but excluded the same municipalities as in Model 2.

To characterize each municipality based on honey yields and bioclimatic conditions, we averaged the pixel values of the corresponding raster maps and added these averages to their centroids (Figure 2). For each modeled dataset we run exploratory data analysis (EDA) to help detect outliers or anomalies in the data. Subsequently, we ran a principal component analysis (PCA) on the correlation matrices both as a way to handle variables that were in different units and to preselect variables based on degree of multicollinearity [47]. To validate this approach, we calculated the variance inflation factor (VIF), a procedure that quantifies the extent of multicollinearity among multiple variables that will be included in regression models [47].

The procedure outlined above helped us eliminate six variables. The remaining 13 variables were included in stepwise multiple regressions to explore the relationship between the log-transformed honey yield and bioclimatic variables (Table 1). Both the Akaike's information criterion (AIC) and the adjusted R-squares are used by Spotfire S+ (TIBCO) to select the model with the best fit. The AIC takes into account statistical goodness of fit while penalizing for increasing the number of variables; low AIC values indicate the model with the best fit [47]. Subsequently, we validated the models generated through the stepwise multiple regression analysis by using the automated model selection function *dredge* in the *MuMIn* package version 1.7.7 of *R* statistical software. This function examines all the possible models given the provided variables and ranks them according to their AIC [48].

2.4. Modeling Honey Yields under Scenarios of Climate Change. Various modeling efforts to predict climate change trends in the Caribbean agree in that the temperature will increase 2°C by 2099 but disagree regarding precipitation

Table 2: Results of stepwise multiple regressions with predictors retained in each of the four predictive models of honey production.

Model parameter	Model 1 (all years and municipalities)	2 (all years but not all municipalities)	3 (yearly average and all municipalities)	4 (yearly average but not all municipalities)
Intercept	3.6328	4.6138	5.1130	6.3048
Isothermality (Bio 3)	—	−0.0099	—	—
Temperature seasonality (Bio 4)	−0.0021	−0.0043	−0.0037	−0.0083
Maximum temperature of warmest month (Bio 5)	—	—	0.1130	—
Minimum temperature of coldest month (Bio 6)	—	—	0.0715	0.0594
Mean temperature of wettest quarter (Bio 8)	−0.0647	−0.0688	−0.2382	−0.1863
Precipitation of wettest month (Bio 13)	—	0.0004	0.0039	0.0012
Precipitation seasonality (Bio 15)	—	—	−0.0285	—
Precipitation of driest quarter (Bio 17)	—	—	−0.0033	—
Precipitation of warmest quarter (Bio 18)	—	—	−0.0009	—
P	<0.001	<0.001	<0.001	<0.001
R^2	0.1903	0.1847	0.5276	0.4984

trends [31–34, 49]. For Puerto Rico, in particular, under the IS92 business as usual scenario (BaU), it was predicted that precipitation would not change during the dry season (DS; December–April) but that it would decrease 20 mm during the early rainy season (ERS; May–July) and increase 15 mm during the late rainy season (LRS; August–November) [32, 50]. In contrast, under the SRES A2 scenario, an ensemble of models predicted that the precipitation was going to decrease 28, 66, and 50 mm during the DS, ERS, and LRS seasons, respectively [31]. We used these two climate change scenarios to model honey yields and modified accordingly the original climate data used as input for BIOCLIM. The new maps depicting these conditions were used as input data to predict honey yields.

We used ArcGIS 9.2 software to create the honey yields and climate spatial databases and to model honey yields across the island under current and future climate scenarios.

3. Results

3.1. Honey Yields. Average honey yields for the period 1998–2005 were estimated at 5.3 ± 4.4 L/colony (mean ± SD) whereas for the historical data this figure was 11.3 ± 9.6 L/colony (t-test, df = 465, $P \leq 0.05$; Figure 1). Contemporary honey yields seem to be less variable (smaller standard deviations (shown above) and have lower maximums (20.5 in 2002 versus 78.7 L/colony in 1910)) than the historical values (Figure 1). Honey yields for the period 1998–2005 as well as the historical dataset exhibit a large intra- and inter-annual variability that may reflect to a large extent differences among the municipalities and time periods in terms of their socioeconomic and ecological potential to sustain honeybee activity. We use the average honey yield (5.3 L/colony) for the 1998–2005 period as a baseline figure to compare the behavior of the models that predict honey yields under current and future scenarios of climate change.

3.2. Modeling Current Honey Yields. The four models differed in terms of the total variance that they explained and the bioclimatic variables associated with honey yields (Table 2). Models 1 and 2 explained the least amount of variance in the data ($R^2 \sim 0.18$) whereas the opposite was true for Models 3 and 4 ($R^2 \sim 0.50$). The models also differed regarding the type and number of variables that were retained with Model 1 and Model 3 retaining 2 and 8, respectively. Four models retained temperature seasonality (Bio 4) and mean temperature of the wettest quarter (Bio 8), three retained precipitation of the wettest month (Bio 13), and two retained the minimum temperature of the coldest month (Bio 6) (Table 2). Honey yields were negatively correlated with temperature seasonality (Bio 4) and mean temperature of the wettest quarter (Bio 8), and positively correlated with minimum temperature of the coldest month (Bio 6) and the precipitation of the wettest month (Bio 13).

Predicted current honey yields varied among the models (Figures 3 and 4, and Table 3). In Models 1 and 3, these ranged between 1.0 and 14.0 L/colony whereas in Model 2 and 4 between 2.0 and 67.0 L/colony, more than doubling the maximum yields predicted by the former (Table 3). The location and extent of the areas suitable for honey yields ≥5.3 L/colony varied among the four models. Models 3 and 4 identified areas suitable for honey production not shown by the other two, and as a result the degree of overlap among the four models was 35%; eliminating Model 4, the most dissimilar model, gave an overlay of 48%. Finally, the predicted areal extent of areas suitable for honey production varied among the models and ranged between 1,000 and 2,200 km^2 or equivalently between 11 and 25% of the total area of the island (Model 1 < Model 3 < Model 2 < Model 4; Table 3).

3.3. Modeling Honey Yields under Climate-Change Scenarios. Under the two scenarios of climate change, the predicted

Table 3: Predicted honey yields and areal extents of suitable honey production areas (honey yields ≥ 5.3 L/colony). Under each model the future climate change scenarios are based on (A) Neelin et al. [31], which follows the A2 scenario, and (B) Angeles et al. [32], the IS92 business as usual scenario.

Model	Predicted honey yields (L/colony)			Predicted areal extents of suitable honey production areas (km^2)		
	Current	Future		Current	Future	
		A	B		A	B
	Max–min (Mean ± SD)	Max–min (Mean ± SD)	Max–min (Mean ± SD)			
1	3.5–14.0 (4.6 ± 0.9)	2.6–10.6 (3.5 ± 0.7)	2.6–10.7 (3.6 ± 0.7)	1,012	221	250
2	3.2–32.3 (5.1 ± 2.1)	2.3–23.7 (3.7 ± 1.6)	2.4–24.2 (3.9 ± 1.5)	1,538	617	704
3	0.6–12.0 (4.4 ± 1.4)	0.2–10.5 (3.4 ± 1.4)	0.6–11.6 (4.1 ± 1.3)	1,370	917	1,149
4	1.7–67.0 (6.2 ± 6.2)	0.9–39.1 (3.5 ± 3.8)	1.0–39.4 (3.9 ± 3.7)	2,210	782	909

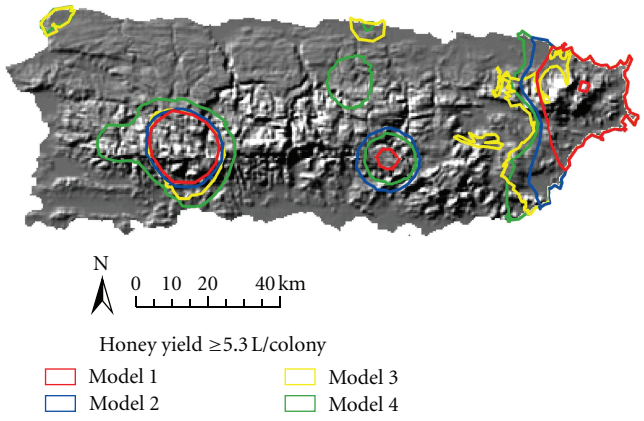

Figure 3: Shade relief map of Puerto Rico showing the predicted current areas suitable for honey yields ≥5.3 L/colony.

honey yields ranged between 10.5 and 39.4 L/colony, which are lower than the predicted current values (Table 3). Yet under the SRES's A2 scenario the minimum and maximum honey yields were slightly lower than under the ISP2's BaU scenario, a result that becomes obvious when examining the areas suitable for honey production (Figure 4; Table 3). All models predicted a reduction in the areal extent of areas suitable for honey production. In the SRES's A2 scenario this reduction ranges between 33% and 78% and under the ISP2's BaU scenario between 16% and 75% (Models 3 < 2 < 4 < 1).

4. Discussion

Average annual honey yields in the island of Puerto Rico were estimated at 5.3 L/colony. The four models developed to predict honey yields under current and future scenarios of climate change varied in terms of the predicted honey yields and the extent and location of areas suitable for honey production (area suitable for honey production; yields ≥5.3 L/colony). The predicted current (minimum and maximum ranges were 0.6–3.5 and 12–67.0 L/colony, resp.) and future (0.2–2.6 and 10.5–39.4 L/colony, resp.) honey yields indicate that climate change has the potential to reduce yields almost by half. In addition, the predicted areal extents of the current (minimum and maximum range between 1,000 and 2,200 km^2) and future (221–1,149 km^2) areas suitable for honey production show a substantial decrease further supporting the likely impact of climate change on beekeeping. Overall these results indicate that climate change has the potential to affect the delivery of provisioning and supporting services by honeybees.

4.1. Honey Yields. Current average honey yields are almost half the historical ones (5.3 versus 11.3 L/colony; Figure 1) and lower than the world (10.7 and 13.1 L/colony for the years 1984 and 1998, resp.) and Caribbean (17.3 and 14.6 L/colony, resp.) estimates ([1]; Food Agricultural Organization (FAO) 1998 database, http://faostat.fao.org/). The long-term trends in honey yields and number of farms with beehives reconstructed from a variety of historical sources resemble a "boom and bust" cycle in which complex interactions between humans, honeybees, and their resource base determine fluctuations in the delivery of ecosystem services (Figure 1; supplementary material). These long-term trends also resemble a population growth model that overshoots its carrying capacity and crashes. In Puerto Rico, a reduction in maintenance research characteristic of modern agriculture [51], in combination with multiple socioeconomic [52, 53] and environmental factors including food availability, diseases, invasions, and natural meteorological events, such as hurricanes, explain these long-term trends and contemporary low honey yields in the island (supplementary material).

Understanding these long-term trends is important because it raises questions about cycles of production and the magnitude of climate change impacts depending on the stage along these cycles. Furthermore, it raises questions about the characteristics of these cycles in developing regions and SIDS (Figure 1).

4.2. Modeling Current Honey Yields. We identified a subset of bioclimatic variables that explains part of the variability in current honey yields, as well as its spatial variability in a tropical region. Three temperature- and one precipitation-derived variables were common to ≥2 models, highlighting the influence of temperature on honey yields. Specifically, honey yields decreased with greater temperature seasonality (Bio 4) and mean temperature of the wettest quarter (Bio 8) in all four models and increased with precipitation of

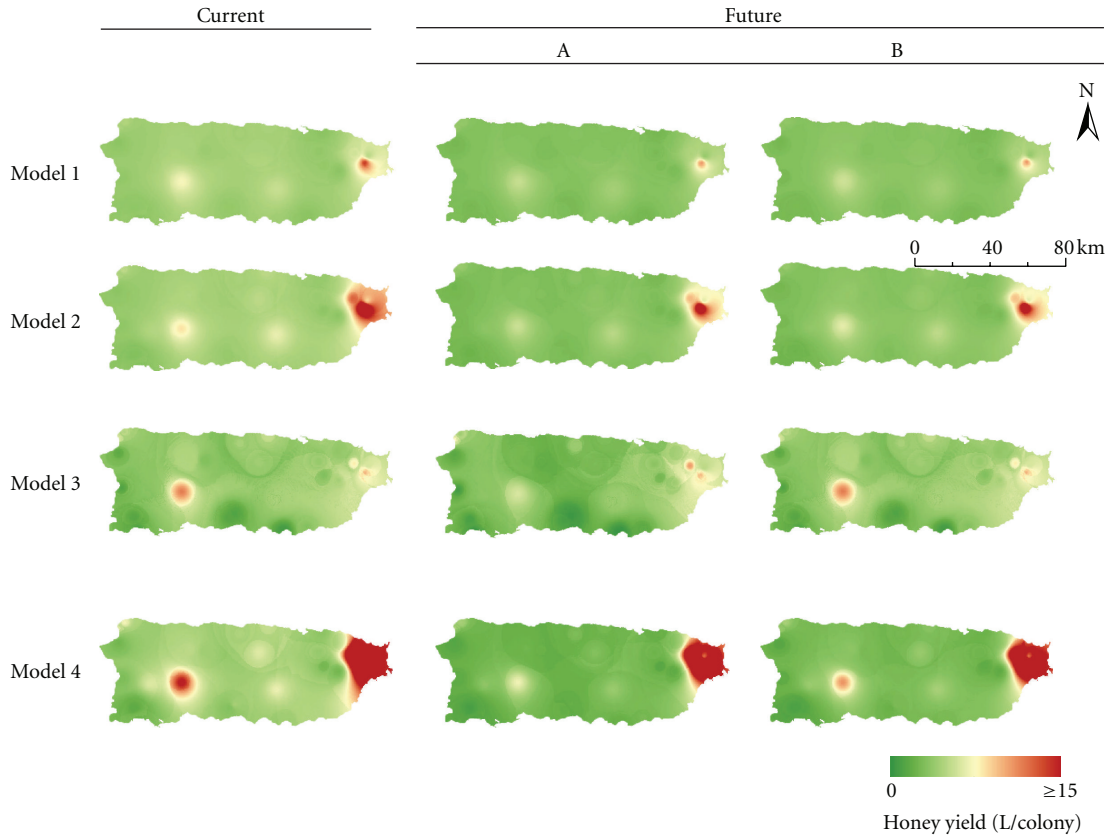

Figure 4: Maps showing the calculated estimates of current honey yield (models 1, 2, 3, and 4) and the honey yields predicted under future climate change scenarios. The future scenarios include a 2°C increase in temperature with a 28, 66, and 50 mm decrease in precipitation during the DS, ERS, and LRS, respectively (Future A) and a 2°C increase in temperature with no change in DS, 20 mm decrease in precipitation during ERS, and 15 mm increase in precipitation during LRS (Future B).

the wettest month (Bio 13) and the minimum temperature of the coldest month (Bio 6) in three and two models, respectively. Although there is considerable variability in these and other bioclimatic variables across the island [35, 37], the general trend is for the wettest quarter of the year that runs from July to September to be a period with the highest maximum temperatures of the year. Likewise, the coldest month is January, a month with some of the lowest precipitations, and the wettest month October, a month in which the high summer temperatures begin to ease. Our results suggest that a combination of extreme temperatures and low precipitation limits the activity of honeybees in this tropical setting.

The map of predicted honey yields identifies regions in eastern Puerto Rico and in the Central Mountains as suitable for average or above average honey yields, which further clarifies the interpretation of our results. These mountainous regions have milder temperatures and are more humid than the lowlands, in particular the dry, low-laying areas of southern and western Puerto Rico [35–39]. Most likely then, the observed variability in predicted honey yields reflects ways in which different life history components of honeybees vary along the complex bioclimatic gradients observed in the island. In Costa Rica, for example, honeybees remain active throughout the year, yet worker brood increased and honey production decreased along an elevation gradient (900–2800 m) located in a mesic environment [15]. This effect, however, was more pronounced during the rainy than the dry season. In Germany, the weight of bee colonies varied across a vast region in Hannover largely in response to the observed variability in climatic conditions and land use [14].

4.3. Modeling Honey Yields under Scenarios of Climate-Change. Honey yields as well as the area suitable for honey production decreased under both climate change scenarios. These results are not surprising given the strong dependency of honeybees on temperature even in tropical regions [5, 14, 15]. More interesting were the effects of precipitation on predicted honey yields. In each of the four models, the minimum and maximum yields did not differ markedly between the two climate change scenarios, but the same was not true for the predicted area suitable for honey production (honey yields ≥5.3 L/colony). In a warmer and drier Puerto Rico, the area will decrease whereas in a warmer and wetter Island the change will be lessened. In Model 3, the model in which several precipitation variables were retained, the area deemed suitable for production was the largest observed. In the model 3b, where there is an overall precipitation increase, this effect was more pronounced. One possibility is that a precipitation increase converts some of the dry

areas of the island into areas suitable for honey production (Table 3, Figure 4).

Results of our work mirror those obtained by others focusing on major food crops delivered by agroecosystems in developing regions and SIDS [25–27]. Specifically it has been shown a decrease in yields towards the tropics [54]. Given the long-term and often complex dynamics of agricultural production, we may ask if climate change in interaction with socioeconomic and environmental factors will contribute to further yield declines [55]. Based on Puerto Rico's beekeeping history we can speculate that the extent to which climate change can impact this and similar agroecosystems including the services that we derive from them may depend upon the stage at which they are found along the "boom and bust" cycle.

4.4. Future Work. We only included bioclimatic variables in our modeling efforts, and the fact that the models explained upto 53% of the variability in honey yields suggests that other variables should be considered. Topography, wind speed, colony management, and land use are logical factors to explore. The first two may influence honeybee behavior as already outlined in our Introduction whereas the third might play a minor role in Puerto Rico's current setting for reasons already mentioned. Land use on the other hand, may integrate characteristics of the food resource base utilized by honeybees, including its quality, quantity, and spatial distribution [6, 14]. It would be worth exploring more mechanistic models that could directly assess the impact of raising temperature and atmospheric CO_2 levels on the physiology of honeybees and their food sources. In particular, raising atmospheric CO_2 levels may impact this agroecosystem in different ways. First, it may increase plant photosynthetic activity and water use efficiency [56], thus increasing the bees' food source base. Second, in some flowering species increases in CO_2 may cause a reduction in nectar production [57], the principal source of food for bees. Lastly, exposure of honeybees to high levels of CO_2 for prolong periods of time can alter insect physiology and behavior, and in some cases it becomes lethal [58, 59].

4.5. Application and Relevance. Beekeeping is promoted as a tool for rural development and conservation not only in the Caribbean but other tropical nations or regions therein ([7, 8]; http://www.beesfordevelopment.org/). Likewise, honeybees are valued for the pollination services provided both to agricultural [4, 60–62] and natural ecosystems, including endangered species [63, 64]. Therefore, any initiative that may increase people's dependency on honeybees in developing regions and SIDS should take into account the likely effect of climate change on beekeeping. Already there are regions where drought is mentioned as the main problem for beekeeping because it leads to shortages of bees' food followed by colony absconding [65]. Equally, shortages of food may increase negative interactions between native bees and honeybees due to niche overlap. Our work is an example of an approach that can provide a better understanding of the bioclimatic factors that limit honey production, and by doing so it may help farmers to cope with new environmental conditions.

5. Conclusions

Contemporary and historical beekeeping records in Puerto Rico revealed a likely reduction in average honey yields, including variability, over time. Current honey yields were used as baseline to compare the behavior of an ensemble of four spatially explicit models that were developed to predict honey yields under current and future scenarios of climate change. The four models varied in their predictions, yet they all showed that honey yields, as well as the area suitable for honey production, will decrease under scenarios of climate change. These results illustrate the possible impacts of climate change on honeybees and ultimately the essential services that they provide to us.

Authors' Contribution

D. L. Delgado Conceived and designed the study, performed the research, analyzed the data, and wrote the paper; M. E. Pérez Analyzed the data; A. Galindo-Cardona conceived or designed the study; T. Giray contributed new methods; C. Restrepo conceived and designed the study, performed the research, analyzed the data, and wrote the paper.

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Research Article

Wild Bumblebee (*Bombus*) Diversity and *Nosema* (Microsporidia: Nosematidae) Infection Levels Associated with Lowbush Blueberry (*Vaccinium angustifolium*) Production and Commercial Bumblebee Pollinators

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The primary objective was to determine if the prevalence of *Nosema bombi* infection is higher for wild bumblebees (*Bombus* spp.) caught in lowbush blueberry growing areas with a history of commercial bumblebee use than for bumblebees caught in areas without a history of commercial bumblebee use. Additionally, we wished to determine relative *Bombus* species abundances and diversity in blueberry growing regions. Over two years we caught, identified to species, and dissected 767 bumblebees. Light microscopy revealed overall infection levels of 5.48%. The history of commercial bumblebee use had no relation to infection levels. Bumblebee species diversity and field location had significant relationships to infection (r^2 adjusted = 0.265; species diversity $F_{(1,22)} = 6.848$, $P = 0.016$; field region $F_{(1,22)} = 5.245$, $P = 0.032$). The absence or presence of one species, *Bombus terricola*, appears to determine the relationship between species diversity and infection. The data show *B. terricola* decline in sampled regions and almost half of the collected *B. terricola* were infected with *Nosema*. The commercial species, *B. impatiens*, shows an increase in abundance, but with a 6.9% proportion infection. Molecular confirmation of the infecting species was ambiguous, suggesting a need for future clarification of the infecting species.

1. Introduction

Native bumblebees (*Bombus* spp.) serve as valuable pollinators for the lowbush blueberry (*Vaccinium angustifolium* Aiton) fields in Eastern Maine and Maritime Canada and Quebec. Typically queens are the predominant foragers during blueberry bloom in Maine [1]. They are known to be effective pollinators due to their ability to forage in cool or rainy conditions [2], which commonly occur during lowbush blueberry bloom in Maine [3], and their use of sonication to remove pollen grains from the *Vaccinium* poricidal anthers [4]. As pollinators of lowbush blueberry, *Bombus* spp. surpass *Apis mellifera* (L.), the most commonly used commercial pollinator, in terms of purity of pollen load (plant fidelity), flower visitation rates, flower handling time, pollen deposition, and percentage of foragers collecting pollen as opposed to nectar [1, 5–7].

Since the early 1990s, commercially reared bumblebees have been available for blueberry growers in Maine [8] and this option appears to be a boon to farmers wishing to enhance pollination by complementing or replacing *A. mellifera*. Furthermore, the species provided in commercial colonies, *Bombus impatiens* Cresson, is technically native to Maine, although it is not known whether the source of commercial genotypes is native to Maine. Many farmers wish to use a native species and many express a hope to populate their fields with subsequent generations of wild bumblebees [9].

The use of commercial bumblebees in Maine lowbush blueberry has not reached the levels of honeybees, peaking

in the mid-2000s, and is since responsible for imported pollination services in about 1.3 to 2.0 percent of the blueberry acreage in Maine [1]. This roughly translates into 400–600 acres of lowbush blueberry fields pollinated with 2000–2400 colonies of commercial bumblebees. These colonies are placed in groups of four, referred to as a quad, directly in the fields where they usually remain until the end of the colony life cycle. Gynes (female reproductives) and males are often produced from the commercial colonies [9].

Consequences of the placement of commercially reared bees in areas with contact to wild bees have been documented [10, 11]. In a greenhouse situation, Colla et al. [12] showed that pathogens were more prevalent in wild bumblebees located near tomato greenhouses using commercial bumblebees than in more distant wild bumblebee populations. In this study, we examine possible consequences of using commercial bumblebees as pollinators of lowbush blueberry. Specifically, we ask: do commercial *B. impatiens* used in the Maine lowbush blueberry agroecosystem, though technically a native bee, impact disease incidence and relative abundances of naturally occurring *Bombus* species?

We chose to answer this question, in part, by looking at the prevalence of the microsporidian genus *Nosema* in wild bumblebees in and around Maine blueberry fields. We expected to find *Nosema bombi* (Fantham and Porter [13]), an obligate intracellular parasite that commonly occurs in North American bumblebees [14]. *Nosema bombi* was one of the pathogens hypothesized by Colla et al. [12] to have jumped from commercial bumblebees to wild bumblebees foraging near greenhouses. It has been suggested that a European strain of *N. bombi* transferred from commercially reared bumblebees has been responsible for the decline of three species of bumblebees [15] including *B. terricola* Kirby, a bumblebee historically found in moderate-to-high population densities in blueberry fields in Maine [16]. Cameron et al. [17] reported *B. terricola* to currently have reduced abundance in relation to historical records while also showing an increased level of *N. bombi* infection (albeit based on a small sample size) in comparison to species without population declines.

The effects of *N. bombi* infection on colony and individual health have proven difficult to assess. Studying laboratory reared *B. terrestris* (L.) inoculated with *N. bombi*, Steen [18] reported low levels of colony success due to poor brood survival of inoculated queens. Likewise, Otti and Schmid-Hempel [19] found infected males had reduced sperm levels and infected queens had decreased ability to mate. In terms of colony success, infected colonies appeared to have reduced population size [20]. In contrast, Whittington and Winston [21] found no significant effects of *N. bombi* infection on colony size (*B. occidentalis* Greene) or amount of brood, although the authors suggest the experimental time (10 weeks) and/or the colony growth limitations due to greenhouse conditions may have obscured effects seen in older or free-ranging colonies. In general, the evidence seems to point to detrimental colony and individual health, (see also [14, 22]) with the understanding that host species [23] and colony genetics [22] might influence the severity of effects due to infection.

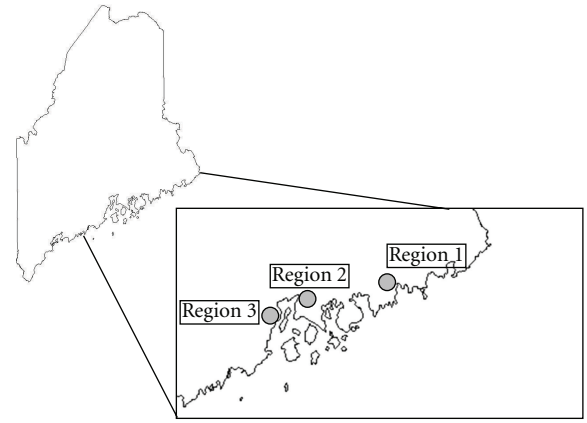


Figure 1: Map of the three lowbush blueberry regions in Maine in which the field sites are located: Region 1: 8 fields, Region 2: 8 fields + 1 unpaired field, and Region 3: 8 fields.

The primary objective of this study was to determine if the prevalence of *N. bombi* infection is higher in bumblebees caught in lowbush blueberry growing areas with a history of commercial bumblebee use than in bumblebees sampled in areas without a history of commercial bumblebee use. Based on the work of Colla et al. [12], we hypothesized that a history of commercial bumblebee use would result in higher levels of *N. bombi* infection. If the history of commercial bumblebee use could not explain the pattern of *Nosema* infection, our second objective was to determine if measurable bee or field characteristics were predictive of infection occurrences. Our final objective was to determine relative *Bombus* species abundances and the species diversity in blueberry growing regions. We hypothesized that due to the use of commercial bumblebees for pollination services, the relative abundance of *B. impatiens* in Maine blueberry growing regions has increased.

2. Materials and Methods

Twenty-four lowbush blueberry fields located in Washington, Hancock, Waldo and Knox counties of Eastern Maine were identified according to their history of commercial pollinator use by personal historical observation or grower interview (Drummond pers. comm.). A field was considered to have a commercial bumblebee history if at any point in time, commercial bumblebees were used in that field for at least one growing season since 1995. A field was considered to not have a commercial bumblebee history if commercial bumblebees had never been placed in that field. Some of the fields without a history of bumblebee use were routinely stocked with honeybees while others were not stocked with commercial bees of any kind, relying instead on wild bee pollinators.

Fields were paired according to the bumblebee history: a yes being paired with a no. The fields were located in three major blueberry regions of coastal Maine (Figure 1), with

each pair located in the same general region. The distance between pairs of fields ranged from 1.5 km to 17.8 km, with a mean distance of 8.9 km. Six pairs of fields were visited for wild bumblebee collecting in 2009 and another independent six pairs of fields were sampled in 2010. All three regions were visited each year. One nonpaired field located in the town of Amherst ME (Hancock Co) that had been previously stocked with commercial bumblebees was visited in 2009.

The fields ranged in size from 2.2 to 20.5 hectares, although five of the fields were contiguous with other blueberry fields that were not included in the study. For these fields, the study collecting area was measured as opposed to total field size, which can run over 500 hectares of continuous blueberry landscape. The remaining collecting sites were isolated, discrete blueberry fields bordered mainly by forest. The collecting area in these fields was the entire forest delineated field.

2.1. Field Management. Production practices varied from field to field. Farmers and field managers were contacted to determine how each individual field had been managed during one complete growing cycle that ended with the year of collection. The fields varied according to the types and extent of pesticide applications and pruning methods (Table 1). Lowbush blueberry pruning methods typically create a two-year cycle with one year of vegetative growth followed by a flowering and then fruiting year. Not all fields were bearing fruit during the collection year.

2.2. Bee Collections. Collection primarily took place in July and August when most foraging bees are workers. This minimizes the capture of queens. At the start of each collection period, a route was planned along field edges where noncrop plants were in flower. In Maine, lowbush blueberry bloom is from mid-May to mid-June. The planned route was covered twice per visit at a steady, slow pace. All observed foraging bumblebees were individually caught with a sweep net and then transferred to a clean 15 mL plastic centrifuge tube. The tubes were stored on ice until the bees could be placed in a freezer (−20°C) where they were held until dissection. In order to best document the diversity of bees foraging in the fields, each field was visited 2-3 times over 4–6 weeks, with a goal of capturing 30 bees per field. In a subset of the fields ($n = 13$), the common name of the flower each bee was caught on was recorded. Some of the common names included several species. For example, "goldenrod" was recorded without differentiating among the possible species. But the recorded names did distinguish the plants at the generic level.

A third year of collecting was conducted in 2011 to develop a relationship between bee abundance, measured as the number of bees collected per unit time and *Bombus* species richness. One hundred twenty-five bees were collected from thirteen new blueberry fields that did not have active commercial colonies of *B. impatiens* or a history of use of such colonies. This collection was conducted in a similar fashion to the two previous years, but the collection bouts were timed. All collecting was done by one person who walked at a regular pace along a predetermined path along flowering vegetation. The collecting took place from 5 August to 19 August. Collected bees were identified to species, but not inspected for *Nosema* infection.

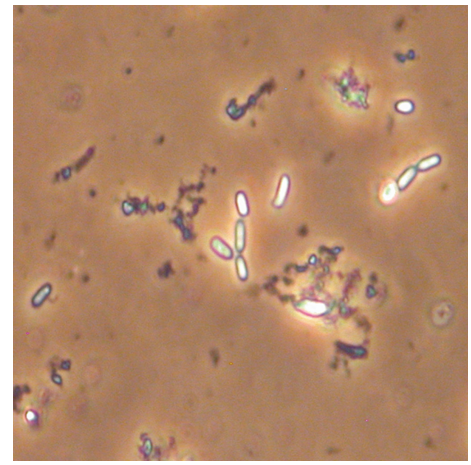


Figure 2: Image of spores classified as *Nosema* by phase-contrast light microscopy. The smaller elongate spores measure 4.5 × 2.0 microns. PCR with the Nbombi-SSU-Jfl/Jrl primer pair did not produce a detectable amplicon for this bee.

2.3. Species Identification, Bee Age, Size, and Sex. All bees were sexed and identified to species using published keys [24, 25] and the online keys available through http://www.discoverlife.org/. For a subset (248 individuals caught in 2009), the right front wing was collected. Using electronic calipers, the length of the marginal cell was measured to 0.01 mm in order to estimate bee size [26]. The degree of wing wear was used to estimate age using a method adapted from Cartar [27].

2.4. Bee Dissection. Each bee collected in 2009 and 2010 was dissected and the gut tissues examined under phase-contrast microscopy at 400x magnification in order to determine if the bee was infected with *Nosema*. A bee was scored positive for infection if two or more microsporidian spores were seen (as in Figure 2). The criteria for spore identification were based on size, shape, and reflectivity as described by MacFarlane et al. [14] and Larsson [23].

The bees were dissected by two different methods. Most of the 2009 bees were opened along the dorsal side of the abdomen. Small (about 2 mm) lengths of the mid and hindgut were removed, placed on a slide in a drop of distilled water and crushed with a coverslip. The remainder of the bee was then refrozen (−20°C). In 2010, the entire abdomen was removed and ground with a pestle in a 1.7 mL microcentrifuge tube with 200 μL of distilled water. A sample of the resulting solution was examined under the phase contrast microscope. This second method of bee dissection was undertaken in order to detect spores that may not have been present in the gut tract but were present in other abdominal tissues [23]. The second method of dissection also prepared the bee for molecular identification of the microsporidian.

Table 1: Production practices associated with the 25 sampled lowbush blueberry fields, 2009-2010. The field was given a score for each production practice based on the treatment the field received for one complete growing cycle (2 calendar years) ending with the bumblebee collection year. These practices were included in the linear model to examine field characteristics and *Nosema* prevalence.

Production practice	Treatment	Number of fields
Pruning	None	3
	Mow	8
	Burn	6
	Mow and burn	8
Pest control	No chemical control	8
	Standard pesticides	4
	Reduced risk pesticides	0
	Standard and reduced risk pesticides	13
Herbicides	Yes	17
	No	8
Fungicides	Yes	15
	No	10
Insecticides	Yes	13
	No	12

2.5. Molecular Confirmation of Infection. All 2010 bee samples that scored positive for *Nosema* with microscopic observations were centrifuged for 5 minutes (16,100 g), the supernatant discarded and the homogenate frozen at −80°C. All of the 2009 *Nosema*-positive and some of the 2009 *Nosema*-negative refrozen dissected bees were thawed and their abdomens removed and similarly ground, centrifuged, and subject to DNA extraction. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen) and amplified using the genus-specific primer SSUrRNA-fl/rlc and species-specific primer Nbombi-SSU-Jfl/Jrl (Table 2) following the protocols of Klee et al. [28] using an Eppendorf thermocycler. The former primer pair contains sequences conserved in *N. bombi*, *N. apis*, and *N. ceranae*, while the later primer is specific to *N. bombi* small subunit rRNA sequences [28]. PCR products were visualized with electrophoresis on a 1.4% agarose gel stained with ethidium bromide. Samples were considered positive for *Nosema* if a band was visible at the expected fragment length (Table 2). Fragment size was confirmed with a 100 bp ladder (Promega). A subsample ($n = 8$) of 222 bp fragments from the PCR products of the genus specific primer SSUrRNA-fl/rlc were extracted from 1.4% agarose gels stained with GelStar (Lonza), purified with a QIAquick Gel Extraction Kit (Quiagen) and sequenced on an Applied Biosystems (ABI) model 377 Sequencer at the University of Maine DNA Sequencing Facility. Edited and aligned sequences were compared with those deposited in GenBank.

2.6. Analysis. For all analyses of relationships between bee host or field factors and infection prevalence, infection prevalence was based on the proportion of bees determined to be *Nosema* positive with microscopic examination. As an indication of possible pathogen spread from a point-source such as one of the fields with a history of bumblebee use, we conducted a Mantel test [29] that compared matrices of differences in field infection prevalence and differences in geographic distances between fields. We used a randomization test with 1,000 iterations [30]. The distances were measured in a straight line from the center of each field using Google Earth (6.1.0.5001). When the most direct route between fields crossed a body of water greater than 1.5 km (a flight distance based on *B. terrestris* L.; [31]) the shortest land route was measured. The closest fields were 1.2 km apart and the most distant were 154.2 km apart. Only the 24 paired fields were used for this analysis. Our question for this test was do fields that are closer together have similar infection levels?

Bumblebee species diversity for each field was calculated as Shannon's index [32]. Because of the difficulty of identifying male bees of the subgenus *Psithyrus* (12 individuals), all bees of this subgenus, including the three females, were grouped and considered one species for diversity calculations. Species richness was defined as the total number of species found in each field. With the 2011 bee species abundance data linear regression [33] was used to develop a predictor of density from species richness so that the effect of estimated *Bombus* spp. density on *Nosema* infection could be assessed ($r^2 = 0.740$; $P = 0.0003$).

A plant generic diversity (Shannon's index, [32]) and generic richness measure (total number of genera represented by the bee catch) were obtained for the subset of 13 fields with known flower types bees were caught on. These measures represent the diversity and richness of the bee-visited floral resources observed in each field and are not an exhaustive list of flowering vegetation, but represent the most common and preferred floral resources. We used linear regression analysis [33] to evaluate the relationship between plant generic richness and diversity measures and *Nosema* infection levels for the thirteen fields.

Considering the full data set of 25 fields, we used stepwise linear regression [33] to select models to examine relationships between 12 field characteristics and field-level (averaged across individual bees) *Nosema* infection levels. The field characteristics included bee species richness, bee species diversity, history of commercial bumblebee use, location (region 1, 2, or 3, see Figure 1), rotational stage (fruit bearing year or not), area, distance to the nearest field with commercial bumblebee use, and the six production practices listed in Table 1. We used a mixed procedure with the probability to leave and enter at $\alpha = 0.250$ and with square root transformed infection proportions. In this model, *Bombus* species richness was used as a proxy for *Bombus* spp. density as described above.

All general statistics and regression analyses were performed with JMP version 8.0.2 [34]. Mantel tests were conducted using PC-ORD, version 6 [30].

Table 2: Primers used for PCR amplification of ribosomal RNA.

Name	Strand direction	Sequence (5′-3′)	Annealing temp. (C)	Expected fragment size
SSUrRNA-fl	Forward	CACCAGGTTG ATTCTGCCT	48	222
SSUrRNA-rlc	Reverse	GTTACCCGTC ACTGCCTTG		
Nbombi-SSU-Jfl	Forward	CCATGCATGTT TTTGAAGATTATTAT	50	323
Nbombi-SSU-Jrl	Reverse	CATATATTTTTA AAATATGAAACAATAA		

Based on Klee et al., 2006 [28], developed from the complete rRNA *N. bombi* consensus sequence, Accession no. AY741110.

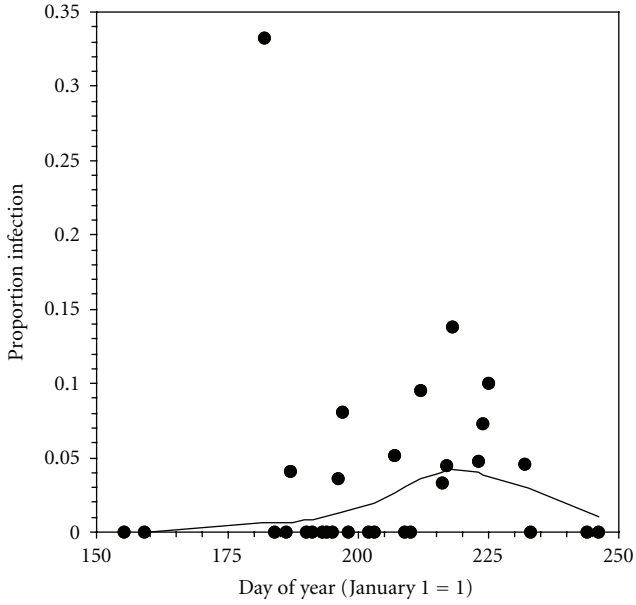


Figure 3: Proportion of bumblebees sampled from Maine blueberry fields showing *Nosema* infection as determined by light microscopy. Proportions shown by day of the year between the sampling period, 4 June–3 September, 2009-2010 (data from two years pooled). The high of 33.33% is from a day with a total catch of three bees, one of which scored positive for *Nosema* spp.

3. Results

Over two years, 767 bumblebees were caught, identified to species, and dissected. Of these, 42 bees were positive for *Nosema* infection according to light microscopy inspection, resulting in an overall 5.48% infection level (Table 3). Neither sex (646 females, 37 female *Nosema* positive), nor dissection method (297 bees by method one, 13 *Nosema* positive), nor year of capture (373 bees caught in 2009 with 16 infected) explained the patterns of infection (Fisher's Exact, $P = 0.66$, $P = 0.33$, $P = 0.21$, resp.). Each bee was given an ordinal rank for the day of the year on which it was caught (Julian day). While not significant at the $\alpha = 0.05$ level ($\chi^2 = 3.48$, $df = 1$, $P = 0.06$) *Nosema* infections showed a trend toward higher incidence as the foraging season progressed (Figure 3), and then declined at the end of the summer. Sampling did not continue in the Autumn.

Table 3: *Nosema* infection in bumblebee species collected in Maine blueberry fields over two years (2009-2010) based on microscopic and molecular examination.

Species	Number positive*/ number caught	% Infection*	Fraction of positive* bees without amplification (see text)
B. terricola	6/13	46.2	0.33
B. perplexus	3/18	16.67	0
B. impatiens	7/102	6.86	0.72
Psithyrus	1/15	6.67	0
B. vagans	10/175	5.71	0.5
B. ternarius	14/374	3.74	0.57
B. bimaculatus	1/68	1.47	1

* Based on visual assessment under 400x magnification with phase contrast microscopy.

3.1. Field History. Of the 25 fields visited for bee collections, 13 were originally identified as having a history of commercial bumblebee use and 12 were identified as no history of commercial bumblebee use. When interviewing growers about management practices, however, one no commercial bumblebee field was changed to a commercial bumblebee field as that grower indicated that a manager of an adjoining field had used commercial bumblebees in the past. This resulted in 14 commercial bumblebee fields (462 bees caught) and 11 no commercial bumblebee fields (295 bees caught). Of these remaining eleven no commercial bumblebee fields, none were located with an adjoining field under different management. Twenty-five *Nosema* positive bees came from commercial bumblebee fields and 17 came from no commercial bumblebee fields. There was no difference in the proportion of infected bees according to the history of using commercial bumblebees (Fisher's Exact Test, $P = 0.87$). This conclusion does not change when considering the original categorization of the fields related to commercial bumblebee use.

Six fields had active commercial bumblebee colonies during the collection periods. There was no indication that those fields had levels of infection that differed significantly from the other nineteen fields (Student's two-tailed test, $t = -0.12$, $df = 23$, $P = 0.45$) or from the 10 fields with no

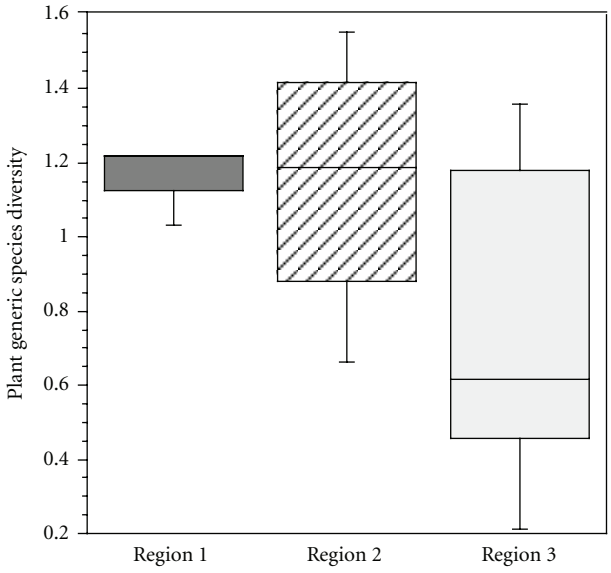


Figure 4: Median, range, and upper and lower quartiles of generic species diversity of plants from which bumblebees were collected along the edge of blueberry fields in three regions in Maine. Based on a subset of 13 fields. Region 1, $n = 3$; Region 2, $n = 4$; Region 3, $n = 6$.

history of commercial bumblebees (Student's two-tailed test, $t = 0.15$, $df = 16$, $P = 0.51$).

3.2. Bee Age, Size, and Species. For a subsample of 248 bees, age and size measurements were obtained. Logistic regression reveals no relation between these two parameters and infection (*size*: Wald's $\chi^2 = .55$, $df = 1$, $P = 0.46$; *age:* Wald's $\chi^2 = 2.57$, $df = 3$, $P = 0.46$; *size*∗*age*: Wald's $\chi^2 = 2.92$, $df = 3$, $P = 0.40$). When considering all 767 bees, it was apparent that infection was not evenly distributed across species (Table 3). The proportion of infected *B. terricola* was significantly higher than the other bee species (Fisher's Exact Test, $P = 0.0002$). We did find one infected individual of the subgenus *Psithyrus*, in contrast to Larsson [23].

3.3. Field Characteristics. For the subset of thirteen fields for which we calculated plant generic diversity and generic richness, we found no significant linear trend relating floral generic richness and field-level infection level ($P = 0.23$). However, plant generic diversity showed a significant, negative relationship with infection level ($F_{(1,11)} = 4.70$, $P = 0.05$) and differed according to collection region (Figure 4).

Out of the 12 field characteristics considered as possible predictors for the occurrence of *Nosema* in the 2009 and 2010 sampling, only *Bombus* species diversity and blueberry growing region were significantly associated with infection. Together, these two characteristics account a little more than 26% of the variation in infection (r^2 adjusted = 0.27; species diversity $F_{(1,22)} = 6.85$, $P = 0.016$; field region $F_{(1,22)} = 5.25$, $P = 0.03$). The proportions of infected bees for collecting regions 1, 2, and 3 were 0.04 ± 0.02, 0.04 ± 0.01, and 0.08 ± 0.02 (mean ± SE), respectively.

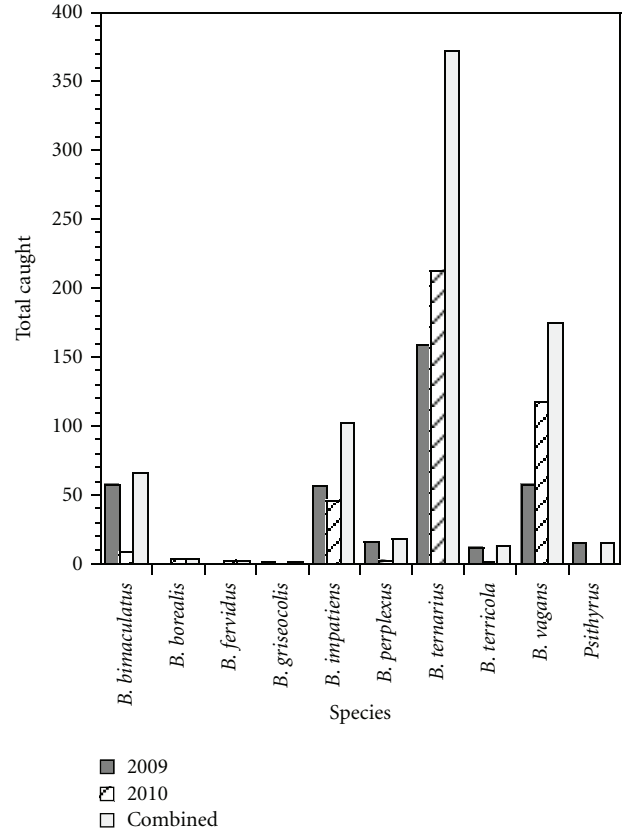
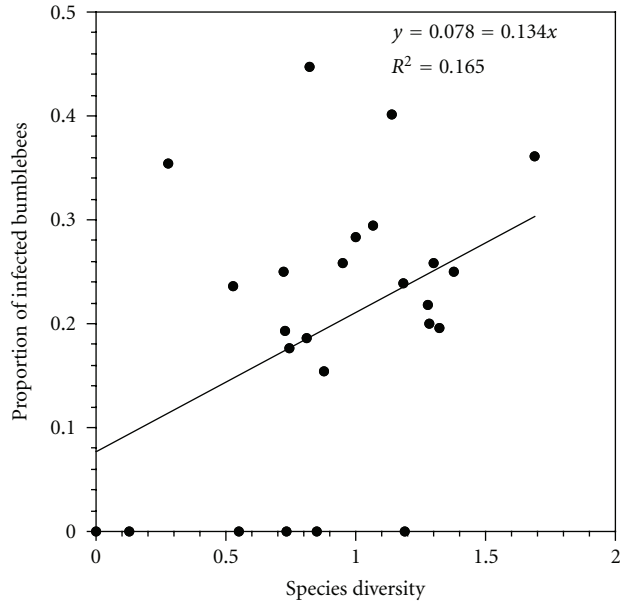


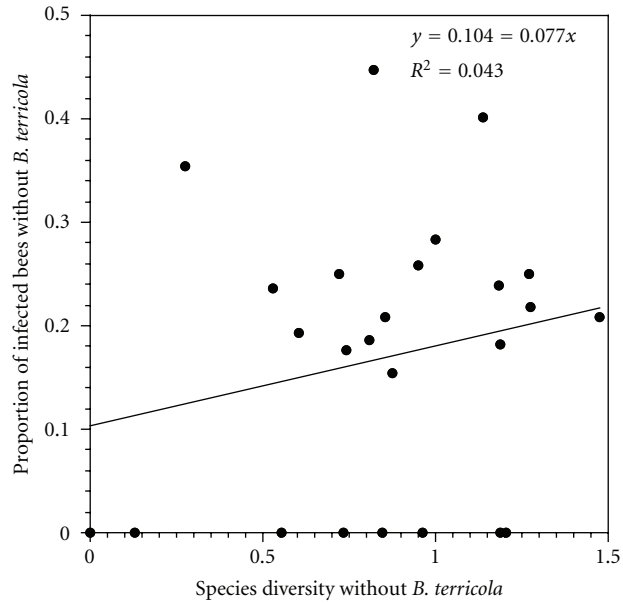
Figure 5: Bumblebees caught in and around lowbush blueberry fields over two years, by year and combined ($n = 767$ bees).

3.4. Bee Species Diversity. Ten species were identified in the total collection, including 15 individuals of the subgenus *Psithyrus* found in five different fields (Figure 5). Figure 5 includes those *B. impatiens* found in fields with active commercial colonies. Although all bees were caught at flowers and commercial bees were not targeted, some of *B. impatiens* (13.3% of total catch) were likely commercial bees. When all *B. impatiens* caught in fields with active commercial colonies were removed from the data set, only 35 individuals remained, which represents 5% of the resulting total. *Bombus ternarius* (Say) was by far the most abundant bee over the two years, making up 48.5% of the total collection and 42.6% and 54.1% of the 2009 and 2010 collections, respectively (Figure 5). The species diversity calculated for the total catch of each year declined over the two years of collecting by nearly 30% (Shannon's Index for 2009 = 1.61; 2010 = 1.14). In order to identify factors that influence species diversity, we compared the relationship between infection and species diversity when *B. terricola* was excluded from the data to the full data set. When all thirteen *B. terricola* caught over two years are removed, the relationship changes from a significant one, to insignificant (Figure 6).

In order to examine long-term trends in relative abundances, we retrieved measures from lowbush blueberry fields from 1961–63 [16] and 1997 and 1998 (Drummond, unpubl. data, $n = 34$ lowbush blueberry fields). The 1960s were well before the widespread use of commercial bumblebees, which



(a) Model: $df = 1, F = 4.56, P = 0.04$



(b) Model: $df = 1, F = 1.02, P = 0.32$

Figure 6: *Nosema* infection (square root proportion infected) of bumblebees relative to species diversity in 25 blueberry fields in 2009 and 2010 with and without *B. terricola*.

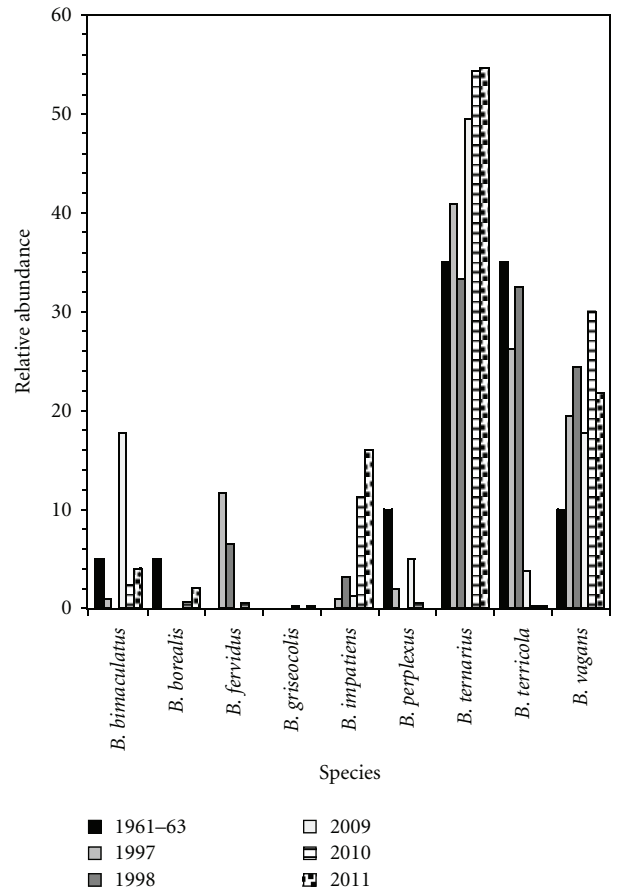


Figure 7: Relative abundances of bumblebee species found in blueberry fields for five years: 1961–63, 1997, 1998, 2009, 2010, and 2011. *Psithyrus* not shown. *B. impatiens* from fields with active commercial bumblebee colonies not included. Data for 1961–63 from Boulanger et al. [16].

were not adopted by Maine blueberry growers until the mid-1990s. We also collected 377 bumblebees in 2011 from blueberry fields within Region 2 of the disease collections. The relative abundances are shown in Figure 7.

3.5. Evidence for Pathogen Introduction. The results of the Mantel randomization test give no indication that fields with similar infection levels are geographical neighbors ($P = 0.16$). A second Mantel test conducted using the logarithm of both variables (proportion infected and distance) gives no indication of a nonlinear relationship between infection levels and geographic similarity ($P = 0.16$).

The molecular confirmation of the infecting species was conducted on 41 of the 42 bees scored as *Nosema*-positive by light microscopy. One bee was omitted from this analysis due to damage during storage. Of these 41 bees, only 21 could be confirmed as being infected with *Nosema bombi* according to our protocol. For these 21 bees, PCR results showed amplification of DNA at the expected fragment lengths for both the general primers designed for detection of *Nosema* spp. (SSUrRNA-fl/rlc) and the primers specific for *Nosema bombi* (Nbombi-SSU-Jfl/Jrl). The remaining 20 bees had no amplification with the species-specific primers. For these bees, however, the results with the more general primers were ambiguous. Amplification products of expected size were present, but often accompanied with fragments of different lengths not associated with the primer. Furthermore, six of the bees scored as negative by light microscopy gave the same result: no amplification with the Nbombi-SSU-Jfl/Jrl primer pair, yet positive, with multiple-bands evident, for the SSUrRNA-fl/rlc primer pair. Of the eight samples sequenced, five sequences were consistent with *N. bombi*,

one consistent with *Nosema* spp., and two gave unreadable results. Of the five consistent samples, four were from the bees confirmed as *N. bombi* and one from those with evident multiple bands with the SSUrRNA-fl/rlc primer pair. The one sample consistent with *Nosema* spp. and the two samples with unreadable results were from those with multiple bands with the general primers. Because of the difficulty of isolating bands at the target fragment length when multiple bands were present (resulting in unreadable results) no other samples were prepared for sequencing.

4. Discussion

The data provide no support for our prediction that the use of commercial bumblebees (*B. impatiens)* in Maine's blueberry fields has increased the prevalence of *Nosema* infection in those fields that have a history of commercial bumblebee use. Although our sample size is low, the power of our test is high, 0.879, for detecting large differences in infection (difference of a 0.5 proportion in *Nosema* spp. prevalence with a minimal detectable odds ratio of 3 and a significance level of 0.05). Therefore, we can conclude that there was no evidence of large differences in prevalence of *Nosema* spp. in wild bumblebees due to the use of commercial bumblebees by farmers. The total infection level of 5.48% does not indicate that coastal areas of blueberry production in Maine have an elevated prevalence of infection. This prevalence is lower than that found in a recent survey of bumblebees in Massachusetts [35], but within the ranges found in multistate surveys [17, 36].

However, when looking at individual species it is apparent that not all species are equally likely to harbor similar levels of infection. Our results show that *B. terricola* has a higher rate of infection than the other *Bombus* species, although our results are based on a low sample size for this species (13 individuals, Table 3). If we assume that infection is independent among collected individuals, then the likelihood of a sample of 13 *B. terricola* (proportion infected 46.2%) coming from a bumblebee community with an average prevalence rate of 5.48% is very low ($P = 0.0000313$, based upon the cumulative binomial distribution). This is a pattern also observed in a recent North American-wide survey [17] and also in a related recent analysis of geographic distributions of *Nosema* [37]. In western North America, *B. terricola* along with two species of the same subgenus, *B. occidentalis* and *B. affinis* Cresson, appear to be declining both in their abundance and range [15, 17, 38, 39]. This decline has been hypothesized to be a result of pathogen spillover from commercial bumblebees [12, 15, 40, 41]. Thorp [15] suggests that in the early 1990s commercial North American bees were reared in Europe and subsequently infected with a virulent biotype of *N. bombi* that was transmitted to wild bees in the US and Canada when colonies from these populations were used for pollination. To the best knowledge of one of us (F. A. Drummond), commercial bees first started being used in Maine lowbush blueberry fields around 1995, a time that would roughly coincide with Thorp's timeline for the introduction of European strains of *N. bombi*. With this study, however, we find no evidence of pathogen spillover when looking at the geographic data set comprising fields from all three regions. Across the blueberry growing regions, we find no clustering of infection. Based on pathogen identification with light microscopy, we find the only suggestion of pathogen spillover is the clustering of the pathogen within one species, which we consider to be insufficient evidence of commercial bumblebee contribution to *Nosema* prevalence in wild bumblebee populations due to the fact that this short-term study is unable to document longer-term disease/host population dynamics.

We found two field characteristics that help explain the distribution of *Nosema* infection across the three blueberry regions. Bumblebee species diversity showed the strongest influence and region of field location as a lesser predictor. We suggest that the species diversity is primarily driven by the presence or absence of *B. terricola* (Figure 6) and that this effect is a result of the nearly 50% infection prevalence of that species.

The fields in the most southern region (Region 3) of our sampling area have a higher mean prevalence of infection than the fields from the other regions. All regions have at least one field with no infected bees, but Region 3 also contains fields with the highest infection prevalence that occurred in the study. The causes of this cluster are not clear. According to our regression analysis that examined twelve field characteristics, only species diversity also explained prevalence of *Nosema* infection. Comparison of species diversity means by region reveals no differences so this measure does not account for the cluster of infections in Region 3. However, the data from the subset of 13 fields did show a negative relationship between plant generic diversity and field infection prevalence. The fields from Region 3 in this subset do show the lowest levels of plant generic diversity found in this study (Figure 4). This could be a spurious relationship, but two factors lower diversity; a small number of species and/or the dominance of a few species in the population. Both of these conditions would force bumblebees to forage on the same flower types, which, if *Nosema* is transmitted on the flower itself, as with other pathogens [42], easier disease transmission between colonies could occur. Further research will be needed to confirm and clarify this relationship.

Our analyses rely on light microscopy to determine the presence of *Nosema* infection in *Bombus*. This approach is limited to identifying pathogens to the genus level only. Spores that were of the correct size and shape for *N. bombi* overlap with those of other *Nosema* species (*N. ceranae*, *N. apis*) [43, 44]. To identify the infecting agent at the species level, we attempted to isolate the pathogenic DNA for molecular analysis. Of the 41 bees showing positive *Nosema* infection via light microscopy and subjected to molecular analysis, only 21 had amplification with the species-specific primer pair Nbombi-SSU-Jfl/Jrl. The other 20 lacked clear amplification, but were clearly infected with *Nosema*-like spores under the light microscope. This may reflect a variant of *N. bombi* which does not amplify with this species-specific primer, or suggest the presence of a different *Nosema* species. The more general primers, SSUrRNA-fl/rlc, amplify conserved regions of rRNA commonly held across *N. bombi,*

N. apis, N. ceranae [28], and other *Nosema* and *Vairimorpha* species. Amplification with PCR from these primers was evident in all 41 bees. Six bees that scored *Nosema*-negative with light microscopy also showed amplification with these primers (but with no amplification with the Nbombi-SSU-Jfl/Jrl primers), a situation which may indicate sample contamination or amplification of nontarget DNA. We cannot conclude, with any confidence, the species of infecting *Nosema* from these PCR results.

The cross-infectivity of *N. apis* and bumblebees has been questioned [23] and while there is evidence that *N. ceranae* has crossed from honeybees to bumblebees in South America [45], such a host jump has not been documented elsewhere. *N. ceranae* appears to have recently crossed from the Asian honeybee (*Apis cerana* F.) to the European honeybee (*Apis mellifera* L.) [44, 46] and is now found globally in infected honeybees [47]. Every year blueberry growing regions of Maine are stocked with rented honeybees, which could prove a source of either *N. ceranae* or *N. apis*. However, given the low likelihood of bumblebees successfully infected with either *N. apis* or *N. ceranae*, and given the known, widespread occurrence of *N. bombi* [17] and lack of evidence of other microsporidian infective agents in bumblebees, we consider it reasonable to treat all observed infective agents as *Nosema* and most likely *N. bombi.* Further research identifying optimal primers in order to reliably sequence isolated gene fragments of the infective agent is warranted.

Exactly half of the 20 bees that did not produce amplification with the Nbombi-SSU-Jfl/Jrl primers came from fields with a history of commercial bumblebee use and half came from fields without the bumblebee use. All collection regions and five species were represented by this group of 20 bees that showed no amplification with the species specific primers. Only two of the originally determined six *Nosema*-positive *B. terricola* are represented by this group (Table 3).

Our final objective was to examine relative species abundances in Maine's blueberry growing region after about 17 years of importation of *B. impatiens* as pollinators. Our recent surveys compared with historic relative abundances (Figure 7) suggest that since the use of commercial bumblebees, the relative abundance of *B. impatiens* has increased. While some *Bombus* species have remained relatively stable, *B. terricola* and to a lesser extent *B. fervidus* (F.), have pronounced reduced abundances. *B. ternarius*, while always abundant, also shows an upward trend. The shift in abundance of *B. impatiens* suggests that queens reared by commercial colonies may be successfully overwintering and founding their own colonies. *Bombus impatiens* was not reported in Maine or Maritime Canada in the 1960s [16], (Drummond unpubl. data). Mark-recapture studies of new queens conducted by Stubbs and Drummond [6] have shown that commercial *B. impatiens* queens will overwinter successfully in Maine. This is not an unreasonable frequent occurrence, as commercial colonies often stay in lowbush blueberry fields through the colony lifecycle. While Figure 7 suggests species shifts within bumblebee communities are occurring, this data does not support or refute the concern that bumblebees as a whole are in decline in Maine lowbush blueberry growing regions as described for other regions globally [48]. However, shifts in biodiversity of bumblebees could have impacts on the ecosystem services provided by these important wild pollinators [49, 50]. This should be a top priority for future research.

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Research Article

Effects of Soil Quality Enhancement on Pollinator-Plant Interactions

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Both biotic and abiotic factors can affect soil quality, which can significantly impact plant growth, productivity, and resistance to pests. However, the effects of soil quality on the interactions of plants with beneficial arthropods, such as pollinators, have not been extensively examined. We studied the effects of vermicompost (earthworm compost, VC) soil amendment on behavioral and physiological responses of pollinators to flowers and floral resources, using cucumbers, *Cucumis sativus*, as our model system. Results from experiments conducted over three field seasons demonstrated that, in at least two out of three years, VC amendment significantly increased visit length, while reducing the time to first discovery. Bumblebee (*Bombus impatiens*) workers that fed on flowers from VC-amended plants had significantly larger and more active ovaries, a measure of nutritional quality. Pollen fractions of flowers from VC-grown plants had higher protein compared to those of plants grown in chemically fertilized potting soil. Nectar sugar content also tended to be higher in flowers from VC-grown plants, but differences were not statistically significant. In conclusion, soil quality enhancement, as achieved with VC amendment in this study, can significantly affect plant-pollinator interactions and directly influences pollinator nutrition and overall performance.

1. Introduction

Mutualisms between flowering plants and animal pollinators are an integral ecological relationship of vital importance for both natural and agricultural ecosystems [1]. Approximately 87.5% of flowering plants use animal-mediated pollination to set seed and fruit [2], corresponding to 70% of our agricultural crops [3]. Therefore, in order to insure the security of our pollinator-dependent crop species, it is imperative to characterize the mechanisms and practices that can enhance pollinator ecosystem services in managed landscapes. However, populations of honey bees, bumble bees, and other native pollinators have been in decline worldwide [4–7]. A reduction in floral resources (quantity and quality) used by pollinators is hypothesized to be a major reason behind this pollinator loss [5, 7, 8]. Thus, there has been increasing interest in understanding how landscape ecology, and, in particular, nutrition provided by flowering plants, can affect and potentially improve pollinator populations in natural and managed ecosystems [9, 10]. Improving nutritional resources for pollinators in agricultural landscapes can be especially important, given that one third of land area worldwide is currently under agriculture management [11] and another billion hectares will likely be converted to agriculture by 2050 as crop production expands to feed a growing human population.

While most studies of plant-pollinator interactions have focused simply on how the plant community can affect pollinator abundance and diversity, the quality of the soil can significantly influence plant-pollinator interactions. Soil quality can influence the production of flowers [12, 13], pollen [14], and nectar [15–17] and can lead to changes in pollinator visitation patterns [12, 13]. However, these previous studies have focused almost exclusively on the impact of the addition of chemical fertilizers to the soil in natural ecosystems, and the outcomes have been variable.

In previous studies, we have demonstrated that vermicompost (VC; earthworm-produced compost) amendment to the soil significantly improves plant resistance to herbivorous caterpillar and aphid species in brassicaceous plant species [18–20]. Here, we sought to extend these investigations to characterize the effects of VC amendment on plant-pollinator interactions. First, we evaluated the effects of VC on cucumber, *Cucumis sativus* L., flower traits over three years. Next, we examined the effects of VC amendment on behavioral responses of pollinators to these plants in the field over three field seasons. We also examined differences between plants grown in VC-treated versus chemically fertilized soils, in the final year of the study, to determine if VC effects were simply due to differences in nutrient availability. Finally, we determined if VC amendment improved the nutritional quality of floral resources, by examining the physiological characteristics of bumblebee (*Bombus impatiens*) workers fed flowers from VC amended versus CF (chemically-fertilized) plants, and by quantifying sugar and protein content in the nectar and pollen of these flowers.

2. Methods

2.1. Plants. Cucumber plants, *Cucumis sativus*, "Boston Pickler" were grown in Sun-Gro Redi-Earth commercial potting mix (CF), or this soil mix amended with 1/3 v : v vermicompost from Oregon Soil Corporation (Oregon City, OR, VC). This level of VC amendment was chosen on the basis of published results [21], as well as plant-insect interactions studies conducted previously [18–20]. Only CF and VC treatments were compared in the 2008 and 2009 field seasons. However, in 2010, we also sought to determine if the differential pollinator attraction to VC-grown flowers documented in 2008 and 2009 was simply due to enhanced levels of major plant nutrients (N, P, K) in VC-amended soil, or if other factors (microbial or chemical) are involved. Thus, in 2010 we also included plants grown in unfertilized potting soil (NF), and plants grown in soil supplemented with a slow release fertilizer (30-3-10 N-P-K; Regal Chemical Company, 600 Branch Dr, Alpharetta, Georgia) with nutrient levels equivalent to those of VC (VCEQ). Levels of these nutrients in VC were determined in a previously published study [20].

Seeds were sown in pairs in 15-centimeter diameter terracotta pots, and seedlings were thinned down to one per pot when they reached the 2nd true-leaf stage. Plants were grown in a greenhouse under natural summer-lighting conditions and 28 ± 5°C and 60–70% RH. All plants were watered 3 times per week with 100–200 mL each.

2.2. Plant Traits. Cucumber plants were used for experiments one week after the onset of flowering (30–35 d after planting). Time to flowering was also noted for each of the treatments. Since pollinator attraction may be influenced by visual factors, such as flower display size [22], the number of flowers and flower weights were obtained at the end of each assay. Additionally, in 2009, we measured corolla diameter and calyx length for sets of ten randomly selected flowers from each of six different CF and VC plants. The measurements for ten flowers were pooled to obtain average calyx and corolla sizes on a per plant basis.

2.3. Pollinator Responses. In 2008 and 2009, assays were run as 2-way choice test between one plant each of CF and VC treatments. In 2010, assays were run as a 4-way choice with all four treatments (NF, CF, VCEQ, VC). In all field seasons, plants were placed in a single row approximately 1 m away from one another. The location of each treatment was rotated for each assay to control for any positional effects. The experiments were carried out at the NCSU campus in an open field, approximately 20 × 20 m, surrounded by native vegetation. Pollinator response was observed during two 15 min intervals, with a 10 minute recess in between. The pollinator number, type (bumblebee, honey bee, or non-bumblebee native bee) and visit length (min) was recorded for each of the treatments. Assays were conducted singly or in duplicates, only on nonovercast days and always between 10 AM and 12 PM. All replicates were obtained between late June and mid August, for a total of 11 replicates in 2008, 14 replicates in 2009, and 8 replicates in 2010.

2.4. Laboratory B. impatiens Microcolony Feeding Experiments. Queenright research colonies of *B. impatiens* L. were obtained from Koppert Biological Systems Inc-USA (Romulus, MI) and were maintained in a room kept at 26 ± 2°C, approximately 60% RH and a 14 : 10 L : D cycle. Colonies were provided with ad-libitum 50% sugar syrup and pollen: honey dough. Fresh food was provided every other day.

Since the field trials showed consistent and significant differences between CF and VC treatments, we compared only these two treatments in this and all subsequent experiments. Given that queenless worker bumblebees can activate their ovaries and initiate egg laying [23–25], and higher levels of dietary protein shorten the time-to-initiate egg-laying and increase egg-laying rates in queenless *Bombus terrestris* workers [26, 27], we examined the effects of feeding queenless bumblebees flowers from CF and VC plants.However, in order to reduce the time required for the experiment and have a detailed measurement of individual bee physiology, we measured ovary activation, rather than egg laying, in three worker bees maintained in queenless micro colonies (see below for details).

All plants were used one week after the onset of flowering. Worker bees were randomly collected from 5 different queenright bumblebee colonies. Insects were weighed individually and sets of three bees of approximately the same weight were placed in 10 × 10 × 7 cm Plexiglas cages (see Figure 1). Each cage was provided with 2 liquid feeders containing 25% sugar syrup. The feeders were made by cutting the bottoms of 1.5 mL plastic micro centrifuge tubes and plugging the openings with rolled up cotton pieces to help wick out the syrup. Syrup in the feeders was replaced every other day. Each cage was also provided with six flowers from CF or VC plants. Fresh flowers were collected daily and their petioles were inserted in 2% agar blocks to keep their turgidity during their time inside the bee cages. Cages were kept for 7 d under

Figure 1: Example of queenless microcolony used to compare effects of VC-grown versus unamended (CF) flowers on *B. impatiens* reproductive physiology.

the same environmental conditions described for the bee colonies above. At the end of the seven days, all bees were killed by freezing. Bees in each cage were collectively weighed and then dissected. A total of 9 replicates were obtained in three trials (different dates) of two, three, and four replicates/treatment each. Data were collected on weight difference (initial-final weights), number of bees showing ovary activation (i.e., ovaries >3 mm long and oocyte development), ovary length, number of oocytes and oocyte length (mean for the four most proximal to the ovipositor).

2.5. Nectar and Pollen Analyses. We used male flowers for sample collection because of their abundance during early flowering, because they were the most predominant during our field assays, and to avoid potential chemical differences due to flower gender. Nectar was collected from individual flowers using 20 μL glass micropipettes to suction all liquid from inside the flower cup. Pollen was collected by manually excising whole anthers from each flower. All flowers were collected between 10 am and noon, and nectar and pollen were obtained immediately following abscission. For each sample, batches of 20 flowers were collected from 2-3 plants in one day. Twelve samples were collected from CF and VC flowers over the course of several days. Corolla diameter and weights of nectar and pollen fractions were recorded at the time of collection. All samples were collected on ice and stored frozen at -80°C until needed for chemical analyses.

Flowers from *Cucumis* spp. have been reported to contain glucose, fructose, and sucrose [28]. Nectar samples were analyzed for these three sugars by high pressure liquid Chromatography (HPLC, Rheodyne/IDEX, Oak Harbor, WA). Nectar samples were diluted using deionized water and passed through a 0.45 um syringe filter. Sugar fraction separation was carried out by on an HPX-87H 30 cm column from BioRad (Hercules, CA) with mobile phase: 0.03 N sulfuric acid. The analytical system was composed of a Thermo Separation Products Refractive Index detector coupled with a P1000 pump set at a flow rate of 0.9 mL/min and Chromquest data analysis software. A four-point calibration curve was obtained with external standards made with high purity, glucose, and fructose to facilitate sugar quantification within each sample.

Pollen samples were thawed and oven dried for 30 minutes to remove excess moisture. To analyze pollen constituents, dried, thawed anthers were extracted by sonication for 50 minutes in pH 7 0.1 M potassium phosphate buffer adjusted to pH 7 with 0.1 M NaOH for protein determination. Approximately 20 mg (weighed to the nearest mg) of dried anthers were extracted in 1 mL of buffer. Samples were then transferred to 1.5 mL microcentrifuge tubes and centrifuged at maximum speed for 10 min to remove particulates. The supernatant was transferred to standard (18×150 mm) borosilicate test tubes for dilution to appropriate concentrations for analysis. In order to read the BCA accurately, extracts were further diluted 1 : 60 with buffer prior to addition of the BCA reagent. Diluted pollen extracts were analyzed for total protein using the standard BCA method (Fisher Scientific, Pittsburg, PA). Extract aliquots of 0.1 mL were added to individual test tubes followed by the addition of 2 mL of BCA working reagent. The tubes were incubated in the dark at 37°C for 30 minutes, samples were then transferred to cuvettes for UV/Vis spectrophotometric (Spectronic Genesys 2, Fisher Scientific, Pittsburg, PA) measurement at 562 nm.

2.6. Statistical Analyses. Because preliminary statistical analysis revealed a significant effect of year, we analyzed and present the results of the different years separately. Therefore, for each of the three years, treatment effects on pollinator response variables such as discovery time (time to first visitor), number of pollinator visits, pollinator type (bumblebee, honey bee, non-bumblebee native bee) and visit length were evaluated with ANOVAs followed by Tukey's mean separation tests. The effect of soil treatment on pollinator visitor type number was evaluated using a Poisson distribution analyses (Proc GLIMMIX) [29], since we would expect each type to be randomly distributed among the treatments. Data for pollinator type number were $\ln(x + 1)$ transformed to comply with the assumptions of normality, but values for means and standard errors in tables are for the untransformed values. Analyses of variance were also used to determine the effects of trial, treatment (CF and VC flowers), and their interaction on the weight difference, survival, ovary length, number of oocytes, and oocyte length in the microcolony feeding experiments. Tukey's mean separation tests were also performed for significant ANOVAs. Differences in flower size between CF and VC plants were evaluated using t-tests (PROC t-test) [29]. The effect of soil treatment on flower weight corolla diameter, pollen and nectar fraction weight, and sugar and protein content were also evaluated using t-tests.

3. Results

3.1. 2008 Field Experiment. Data for flower traits and pollinator responses are provided for each year on Tables 1 and 2, respectively. In 2008, number of flowers per plant did not

Table 1: Effect of soil treatment on cucumber flower traits in field assays. Two-way choice in 2008 and 2009 included CF (potting soil plus Osmocote 14-14-14 at the label rate) and VC (potting soil plus 33% vermicompost) treatments. Four way choice field assays in 2010 included CF, VC, NF (potting soil without fertilizer), and VCEQ (potting soil plus slow release fertilizer with equivalent NPK as those provided by vermicompost) treatments. Values present means ± SE from 11, 14, and 8 replicates for each season, respectively. Means within rows followed by the same superscript letter are not significantly different (Tukey's mean separation test, $P > 0.05$).

Year	Response variable	CF	VC	NF	VCEQ
2008	Number flowers	9.6 ± 0.87^{a}	9.0 ± 0.41^{a}	NA	NA
	Flowering time (d)	30 ± 0.50^{a}	27.8 ± 0.52^{b}	NA	NA
	Flower weight (g)	0.2 ± 0.01^{a}	0.23 ± 0.00^{b}	NA	NA
2009	Number flowers	12.8 ± 1.44^{a}	12.8 ± 1.28^{a}	NA	NA
	Flowering time (d)	30.7 ± 0.22^{a}	29.4 ± 0.17^{b}	NA	NA
	Flower weight (g)	0.23 ± 0.01^{a}	0.25 ± 0.01^{b}	NA	NA
2010	Number flowers	$9.8 \pm 0.1.36^{b}$	6.3 ± 0.41^{a}	4.6 ± 0.29^{a}	5.1 ± 0.33^{a}
	Flower time (d)	NA	NA	NA	NA
	Flower weight (g)	0.25 ± 0.01^{b}	0.32 ± 0.01^{c}	0.23 ± 0.01^{ab}	0.20 ± 0.01^{a}

Table 2: Effect of soil treatment on pollinator responses to cucumber plants in field assays. Two-way choice in 2008 and 2009 included CF (potting soil plus Osmocote 14-14-14 at the label rate) and VC (potting soil plus 33% vermicompost) treatments. Four way choice field assays in 2010 included CF, VC, NF (potting soil without fertilizer), and VCEQ (potting soil plus slow release fertilizer with equivalent NPK as those provided by vermicompost) treatments. Values represent means ± SE from 11, 14 and 8 replicates for each season respectively. Means within rows followed by the same superscript letter are not significantly different (Tukey's mean separation test, $P > 0.05$).

Year	Response variables	CF	VC	NF	VCEQ
2008	Discovery time	10.3 ± 2.68^{a}	7.7 ± 1.86^{a}	NA	NA
	Number Visits	8.3 ± 1.68^{a}	9.1 ± 0.86^{a}	NA	NA
	Visit length (min)	1.1 ± 0.32^{a}	1.9 ± 0.29^{b}	NA	NA
	Bumblebee (%)	66.4 ± 8.25^{a}	65.4 ± 8.67^{a}	NA	NA
	Honey bees (%)	11.7 ± 5.79^{a}	8.0 ± 4.5^{a}	NA	NA
	Other native bees (%)	21.8 ± 9.53^{a}	26.4 ± 9.26^{a}	NA	NA
2009	Discovery time	7.7 ± 1.84^{a}	3.92 ± 1.05^{b}	NA	NA
	Number Visits	13.4 ± 1.72^{a}	15.6 ± 1.88^{a}	NA	NA
	Visit length (min)	1.3 ± 0.14^{a}	2.1 ± 0.24^{b}	NA	NA
	Bumblebee (%)	94.3 ± 32.82^{a}	81.0 ± 27.17^{a}	NA	NA
	Honey bees (%)	0 ± 0.0^{a}	0 ± 0.0^{a}	NA	NA
	Other native bees (%)	5.6 ± 1.76^{a}	18.9 ± 3.38^{b}	NA	NA
2010	Discovery (min)	20.5 ± 3.96^{b}	13.9 ± 3.81^{c}	43.4 ± 1.62^{a}	19.6 ± 3.61^{b}
	Number Visits	3.6 ± 1.26^{a}	7.0 ± 2.22^{a}	3.1 ± 1.20^{a}	3.0 ± 1.41^{a}
	Visit length (min)	0.18 ± 0.07^{b}	0.67 ± 0.23^{c}	0.05 ± 0.08^{a}	0.20 ± 0.08^{b}
	Bumblebee (%)	43.3 ± 16.75^{a}	24.2 ± 11.66^{a}	22.8 ± 9.72^{a}	48.9 ± 18.52^{a}
	Honey bees (%)	19.2 ± 12.31^{a}	26.1 ± 9.99^{a}	26.9 ± 12.71^{a}	13.5 ± 12.39^{a}
	Other native bees (%)	12.5 ± 12.50^{a}	49.6 ± 15.70^{b}	12.8 ± 8.31^{a}	12.5 ± 12.50^{a}

differ between treatments, but VC plants flowered significantly earlier ($F = 3.81$; df $= 1, 29$; $P = 0.033$), (Table 1) and had significantly heavier flowers ($F = 49.94$; df $= 1, 29$; $P < 0.0001$), (Table 1).

Time to first discovery, although shorter for the VC plants, was not statistically different (Table 2). However, the pollinators spent significantly more time on the VC flowers ($F = 6.86$; df $= 1, 29$; $P = 0.0035$), (Table 2). The number of visits and pollinator type was comparable between the treatments though there was a tendency for more visits to VC plants (Table 2). Pollinator types were bumblebees, honey bees and nonbumblebee native (Megachillidae with an occasional visit by *Peponapis* squash bees, *Xylocopa* carpenter bees, Hesperidae butterflies, and Syrphidae flies).

3.2. 2009 Field Experiment. We again compared VC and CF plants in these experiments. Flower corolla diameter and calyx length did not differ significantly between CF and VC plants. Corolla diameter mean ± SE in cm was 4.6 ± 0.19 for VC and 4.5 ± 0.16 for CF plants. Calyx length mean ± SE in cm was 0.69 ± 0.007 for VC and 0.69 ± 0.012 for CF plants. Similar to 2008, the number of flowers was not significantly

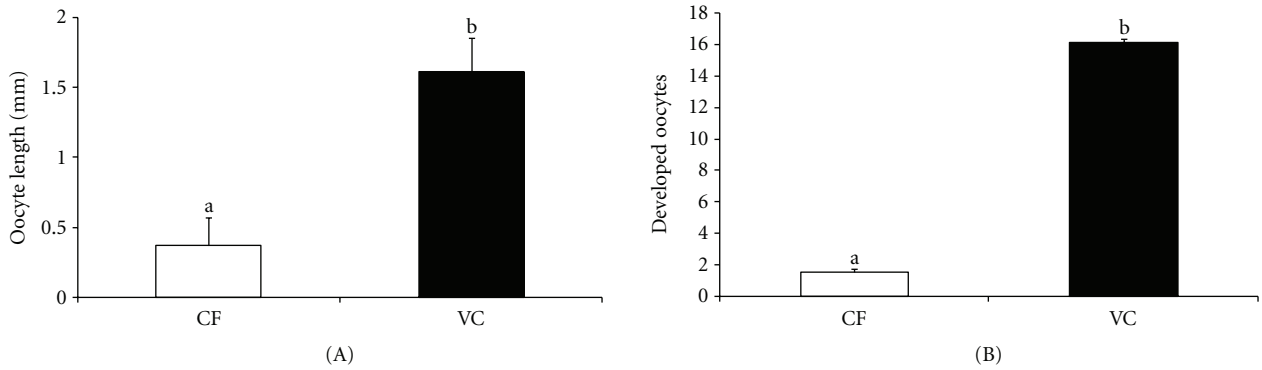

Figure 2: Effect of feeding on flowers from cucumber plants grown in unamended (CF) soil and VC-amended soil on (A) Oocyte length and (B) Developed oocyte number. Values represent means for 9 replicates and error bars are equivalent to 1SE. Bars headed by the same letter are not significantly different, Tukey's mean separation test ($P > 0.05$).

Table 3: *Bombus impatiens* survival, weight, and ovary length of bees in queenless micro-colonies fed flowers from plants grown on CF or VC-amended soil. Values represent means ± SE for 9 replicates. Means within rows followed by the same superscript letter are not significantly different (Tukey's mean separation test, $P > 0.05$).

Response variable	CF	VC
Survival (%)	81.6 ± 7.99^{a}	96.3 ± 6.33^{a}
Weight (mg)	371 ± 19.8^{a}	426 ± 25.0^{a}
Ovary length (mm)	3.4 ± 0.77^{a}	5.0 ± 0.41^{b}

different (Table 1); time to first flowering was again shorter in VC plants compared to CF plants, but this was marginally significant ($F = 3.92$; df $= 1, 46$; $P = 0.050$), (Table 1); and flower weights were also larger for VC plants ($F = 6.72$; df $= 1, 26$; $P = 0.0390$), (Table 1).

Time to first discovery was significantly different between treatments, with pollinators finding VC plants faster than CF plants ($F = 4.42$; df $= 1, 50$; $P = 0.0454$) (Table 2). There was also a significant effect on pollinator visit length ($F = 6.24$; df $= 1, 50$; $P = 0.0345$), with insects spending more time on VC versus CF flowers (Table 2). Number of pollinator visits was not significantly different between treatments, though tended to be higher for VC compared to CF plants (Table 2). Similar to 2008, bumblebees were the predominant pollinators in both treatments (Table 2), but this was not significantly different between treatments, while honey bee visits were negligible (Table 2). There were, however, a significantly higher proportion of non-bumblebee pollinator visitors to VC versus CF flowers ($F = 17.76$; $P = 0.0006$), (Table 2).

3.3. 2010 Field Experiment: Soil Nutrient Effects on Plant and Pollinator Responses. The number of flowers was significantly different among the treatments with NF plants having higher numbers compared to all other treatments ($F = 9.59$; df $= 3, 28$; $P < 0.0001$), (Table 1). Time to first flowering was not recorded in this season. Flower weight was highest for the VC, followed by CF and NF treatments and lowest for the VCEQ treatment ($F = 13.08$; df $= 3, 28$; $P < 0.0001$; Table 1).

Both plant discovery time and pollinator visit length were significantly different among the treatments (Table 2). Plant discovery time by pollinators was significantly lower ($F = 14.72$; df $= 3, 28$; $P < 0.0001$) while pollinator visit length ($F = 14.72$; df $= 3, 28$; $P < 0.0012$) was found to be significantly higher for VC compared to all other treatments (Table 2). Number of pollinator visits to each of the treatments did not differ significantly, although there was a tendency for the VC plants to have a greater number of visitors compared to all other treatments (Table 2). Similar to the 2009 season there was also a significant ($F = 15.56$; $P = 0.0016$) increase in the mean number of native non-bumblebee pollinator visits to VC compared to all other treatments (Table 2). Again, the predominant pollinator species visiting the flowers were bumblebees (Table 2). Unlike the previous field seasons, honeybees were the second predominant pollinators (Table 2). Non-bumblebee native pollinator responses were negligible for NF, CF, and VCEQ, but interestingly, they comprised a significant portion of responders to the VC treatments (Table 2).

3.4. Laboratory Bumblebee (B. impatiens) Microcolony Feeding Experiments. Trial and its interaction with treatment were not found significant for any of the variables tested. Also, VC versus CF treatments did not significantly affect the weight difference (though there was a tendency for VC bees to be heavier) or survival of bees in queenless microcolonies (Table 3). However, ovary length ($F = 4.9$; df $= 1, 16$; $P = 0.0417$), (Table 3), number of oocytes ($F = 22.6$; df $= 1, 16$; $P = 0.0002$; Figure 2(A)), and oocyte length ($F = 16.6$; df $= 1, 16$; $P = 0.0009$), (Figure 2(B)) were all found to be significantly increased in bees fed flowers from VC plants. It should also be noted that more bees in the CF-fed cages (2.1 ± 0.26) displayed lower levels of ovary activation than in the VC-fed cages (1.3 ± 0.23)

suggesting that the bees in the CF cages were in the early stages of establishing reproductive dominance hierarchies, and a clear alpha dominant reproductive worker (which suppresses ovary activation in subordinate workers) had not yet emerged.

3.5. Nectar and Pollen Analyses. There were no significant differences in corolla diameter or nectar and pollen weights between the CF and VC flowers used in this assay (data not shown). However, protein content was significantly higher in VC pollen fractions versus CF (34.5 ± 2.24 versus 26.1 ± 1.23 mg/g dry mass, respectively, $t = 3.22$; $P = 0.0062$). Sugar content was slightly higher in the nectar of VC flowers versus CF, though this was not significant (96.9 ± 10.13 and 98.5 ± 9.10 mg/mL).

4. Discussion

Previous studies have found that soil enhanced with VC amendment can affect plant-pest interactions [18–21, 30, 31]; however, data presented herein demonstrates for the first time that it can also impact plant interactions with beneficial arthropods. Overall, soil VC amendment caused significant increases the time pollinators spent on flowers (in all three years of the study) and significantly lowered time to plant discovery by pollinators (in two of the three years). Soil VC amendment also increased pollen protein content, which correlated with increased ovary activation in bumblebee workers, a trait dependent on protein nutrition. These results suggest that soil quality improvement, as demonstrated by VC amendment in this study, can positively affect interactions with pollinators and provide a higher quality food source for these insects.

As with previous studies [30–32], we found several aspects of plant phenotype modulated by VC soil conditioning. Number of flowers did not differ statistically between CF and VC in 2008 and 2009 but were significantly lower for VC in 2010. Yet, VC plants had heavier flowers than CF in all three years. Flower weights were also significantly higher for VC plants compared to VCEQ and NF in 2010. Additionally, VC plants flowered significantly earlier than the CF in the two years this trait was examined (though note that the 2009 results were marginally significant, with a P value of 0.050).

Amendment of soil with VC also significantly altered pollinator interactions with flowers, though the pollinators visiting plants in our study are similar to those reported for cucumber systems by other authors [33]. Pollinators spent more time on the flowers of VC plants compared to those of CF or VCEQ plants in all three years. Time to plant discovery was significantly shorter for VC compared to CF plants in 2009 and 2010, but this was not significantly different in 2008. Pollinators use a variety of cues to identify high-quality flowers, primarily visual but also olfactory [25, 33], and this suggests that one or both of these cues may be altered by the soil amendment used in this study. Given that flowers did not differ in size and the number of flowers was in some instances (2010) lower for the VC plants, simple flower-associated visual cues cannot account for the pollinator responses observed. Thus, differences in volatile cues are likely behind the faster plant discovery and enhanced attraction/arrestment observed herein. Buchmann and Cane [34] found that pollinating bees were able to detect pollen level availability in the flowers of *Solanum* and increase their visitation time and number of sonicating buzzes accordingly. More interestingly, bumblebees collecting pollen from *Dodecatheon conjugens* and *Lupinus sericeus* were shown to detect and respond to differences in pollen availability, even though the plant species tested concealed pollen from view [34, 35]. This indicates that nonvisual cues can in fact influence the pollinator responses to higher quality flowers. Interestingly, in the 2009 and 2010 field assays there was higher proportion of visitations to flowers of VC plants by non-bumblebee native pollinator species, suggesting that the cues produced by these plants are more appealing to these pollinator types.

Our studies also demonstrated that plants grown with VC produce more nutritious floral rewards for pollinators. We found that bumblebee workers that fed on VC-flowers had larger and more developed oocytes than bees fed on CF-flowers. Because bumblebee worker reproduction is dependent on dietary protein content [26, 27], these results suggest that the nutritional quality of the floral resources produced by the VC-treated plants are higher and thus allowed for more rapid and complete ovary activation in the queenless bumblebee workers. Indeed, chemical analyses demonstrated that protein content of the pollen fractions was significantly higher in VC versus CF plants. It is also worth noting that overall difference in sugar content was an average of 1.6 mg/mL higher in VC plants, and, although statistically insignificant, such an amount of sugar may still be biologically relevant for pollinators.

Previously published research on soil fertilization on plant-pollinator interactions has yielded variable outcomes. For example, soil phosphorus was shown to influence the size of a pollen grain and its chemical composition, which enhanced pollinator-dependent male flower reproductive success [14]. Baude et al. [36] investigated the effect of N provision through soil litter amendment on plant performance and pollinator resources (nectar sugar) and found disparate, plant-species-dependent responses to the treatments. In other studies, Burkle and Irwin [13, 17, 37] evaluated the impacts of N fertilization on plant-pollinator interactions in a subalpine natural ecosystem, and found no effects of N enrichment on the diversity or visitation rate per flower by pollinators, even though floral biomass and seed production responded positively to N supplementation. Hoover et al. [38] reported that when soil was amended with a simple nitrogen fertilizer, pollinators were not significantly more attracted to the nitrogen treated plants, and, interestingly, bees fed nectar from nitrogen-treated plants had higher mortality. Results from our studies provide further evidence that the effects of VC soil amendment on plant flower traits and pollinator interactions are not simply due to changes in nutrient (N, P, K) availability. In the 2010 studies, VC plants had significantly heavier flowers, had longer pollinator visits, and, though not significant, tended to attract more pollinators than plants grown in soil with VCEQ nutrient levels. Therefore, non-nutritional factors associated with VC amendment, such as microbial interactions, physical

properties, or non-nutrient chemical compounds, may be responsible for enhancing floral resources. Further studies examining the biotic and abiotic factors associated with VC amendment are necessarily to further understand this system.

Our studies indicate that, in addition to previous documented benefits of VC soil amendment on plant growth, productivity and resistance to diseases and pests [18–21, 39, 40], these treatments also enhance plant pollinator attraction and visit length and result in better quality food sources for pollinators. We chose a native bumblebee species to use as a model system for this project due to their small colony size and their predominance as pollinators of cucumber plants. However, we feel certain that the methodology and results obtained are transferable to other pollinator species, as is evident in the responses of non-bumblebee native fauna recorded in this study. These results yield critical information on how soil organic amendments influence aboveground plant symbiotic interactions, and how these could be manipulated to increase ecological services (i.e., pollination) for crop production. Nonetheless, further studies need to be performed to assess field-level applicability of this resource, based on overall arthropod (both pest and beneficial) community responses, cost feasibility, and overall crop performance.

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Research Article

Efficiency of Buzzing Bees in Fruit Set and Seed Set of *Solanum violaceum* in Sri Lanka

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Plant-pollinator interactions are often considered as tightly coevolved, mutualistic relationships. The present study aimed at determining the flower visiting bees of the vegetable crop, *Solanum violaceum*, and the efficiency of buzz pollination by bees on fruit and seed production in Sri Lanka. Seven bee species: *Hoplonomia westwoodi*, *Amegilla comberi*, *Patellapis kaluterae*, *Xylocopa tenuiscapa*, *Apis dorsata*, *Trigona iridipennis*, and *Ceratina hieroglyphica* visited the flowers of *S. violaceum*, and the first four species were buzzing bees. Buzzing bees were the first to visit *Solanum* flowers and were followed by nonbuzzing bees. Handling time of *H. westwoodi* and *P. kaluterae* varied with the availability of pollen in anthers that deplete with the age of flower and stayed longer at new flowers than at old flowers. Handling time of the larger buzzing bee, *H. westwoodi*, was higher than that of the smaller *P. kaluterae*. The fruit set, seed set, and seed germinability in flowers visited by buzzing bees were significantly higher than those of the flowers bagged to exclude pollinators.

1. Introduction

Plant-pollinator interactions are very complex [1] and nearly three-quarters of Angiosperms rely on animal vectors to move pollen among flowers [2]. Colour, shape, and odour are well-known characteristics of flowers which partly determine the types of animal pollinators that visit them [3]. Unlike the majority of flowering plants in which the anthers open by splitting along the entire locule, many unrelated plants displays an unusual anther rupture mechanism in which certain anthers are poricidally dehiscent [4]. Anthers of 72 families and 574 genera of the flowering plants species dehisce via pores and of them 54 families and 357 genera restrict pollen removal by buzzing bees [5]. Pollen removal requires bees that land on the flowers [6], curl around the "anther cone" [4], and vibrate their indirect flight muscles at high frequency in contact with anthers and thereby induce rapid pollen liberation [7]. This produces an audible buzzing sound and is a unique form of pollination termed "buzz pollination" [5].

Many bees including solitary and social species, and both generalists and specialists routinely use sonication to harvest pollen [8]. Sonicating bees are found in most of the major bee families in the world, in at least seven families and over 50 genera [9]. Buchmann and Cane [8] further stated that the two genera, *Apis* and *Trigona* have never been observed to sonicate poricidal flowers. Bees belonging to the genera *Hoplonomia, Lasioglossum*, *Patellapis* (family Halictidae), and *Amegilla* and *Xylocopa* (family Apidae) have been observed to buzz at flowers with poricidal anthers in Sri Lanka [10].

Buzzing bees are usually active in early morning because anther dehiscence of most buzz flowers occurs during this period. The total time of vibration of anthers of a given flower by a buzzing bee is termed the handling time of the bee [5]. The handling time differs from one bee species to another [11]. Buchmann and Cane [8] found a positive relationship between pollen availability and handling time for individual floral visits, indicating immediate assessment of pollen returns by bees in flowers of *Solanum elaeagnifolium.* Furthermore, they have observed bees selectively visiting younger pollen-rich flowers than older flowers spending more time on younger flowers. Solanaceae, Melastomataceae, Bixaceae, Cochlospermaceae, Fabaceae, and Dilleniaceae are

few examples of plant families that are pollinated by buzzing bees [12].

The dehiscence of anthers through two small apical pores is a feature found in many species of Solanaceae and especially in the genus *Solanum* [13]. *Solanum* flowers provide a relatively rich pollen source for bees that visit them [14]. Although they lack nectar and restrict access to pollen (having only terminal anther pores), they are heavily visited by a large number of individuals of at least a few species of bees [15]. *Solanum* is a cosmopolitan genus of more than 2000 species and is the second largest genus of flowering plants [8]. The genus *Solanum* is of worldwide economic importance, including major crop species such as *Solanum melongena* (eggplant) and *S. tuberosum* (potato). Even if the general syndrome of *Solanum* pollination is well known, there is little information [11, 16] about specific pollinators and pollination of *Solanum* species [15]. *Solanum violaceum* is a delicate perennial species often cultivated as an annual shrub. *Solanum violaceum* is considered as a wild plant in most of the world, but is a vegetable crop in Sri Lanka. However, the fruits are mostly collected from wildly grown shrubs and are very expensive in the local market. No studies have been conducted so far to identify the wild pollinators of *S. violaceum* and their importance in fruit and seed set. Therefore, the present study was designed to (i) determine the time of stigma receptivity and anther dehiscence in *S. violaceum* flowers, (ii) identify the bees that collect and carry pollen of *S. violaceum* and record their activity period, (iii) investigate the handling time of different bee species at *S. violaceum* flowers at different age (during peak activity period of bees), and to (iv) assess the fruit set and seed set in bagged flowers to prevent insect visits and open flowers that receive insect visitors.

2. Materials and Methods

The study was conducted in two sites: one located in Meewatura; Agriculture Research Field 7°15′N, 80°45′E) in the Peradeniya University Park in the Kandy district and the other in a home garden (7°15′11″N, 80°21′2″E) in the Kegalle district. Pollination trials were conducted only in the field at Kegalle where 25 seedlings were cultivated for the experiment.

2.1. Determination of the Time of Stigma Receptivity and Anther Dehiscence. Time of stigma receptivity and anther dehiscence was observed in freshly opened five flowers of *S. violaceum*. The time of stigma receptivity was investigated by observing the stigma through a hand lens at every 10 minutes from 6.30 a.m. to 2.00 p.m. The stigma was touched by a needle tip to observe the stickiness and was considered as the time of stigma receptivity. The time of anther dehiscence was observed by shaking the flower onto a white paper every 10 minutes from 6.30 a.m. The time at which pollen was released and collected onto the white paper was considered as the time of anther dehiscence.

2.2. Determining the Number of Pollen Grains in Anthers of Flowers at Different Age. Flowers at different age, new, 1-day-old, 2-day-old and >2-day-old flowers were selected. From each flower, one anther was removed and placed in a solid watch glass. The anther was dissected longitudinally and the pollen grains were removed into the watch glass. One milliliter of 50% alcohol was added into the solid watch glass containing the pollen grains. From this mixture, 1.00 ml volume was transferred on to a Sedgewick-Rafter Cell (a hemocytometer). Five cells with high amount of pollen grains were counted and the average number of pollen grains in the chamber was estimated. This was repeated for flowers at different age using five anthers at each age category.

2.3. Collection and Identification of Bees Visiting S. violaceum Flowers. Bees visiting flowers of *S. violaceum* were collected using a sweep net. At the Kegalle site, flower visiting bees were observed for ten sunny days until no new species were recorded. Buzzing bees were identified by the audible buzzing sound they produce at anthers during pollen gathering from anthers. Bees that do not produce an audible sound at anthers were grouped as nonbuzzing bees. Bees were collected from May 2009 at Meewature site where the preliminary survey was conducted and from August to November 2009 at Kegalle site. Bees were identified using keys to identify bees of Sri Lanka [17] and reference collection of bees lodged at the invertebrates systematics and diversity facility (ISDF) in the Department of Zoology, University of Peradeniya.

2.4. Recording of Activity Time of Buzzing and Nonbuzzing Bees Visiting Solanum Flowers. The activity time of both buzzing and non-buzzing bee species were observed at the Kegalle site. The activity time of bees were determined by their visits to flowers between 7.00 a.m. to 4.00 p.m. on four sunny days. The abundance of each bee species was not determined.

2.5. Determining the Handling Time of Two Common Buzzing Bees on Flowers at Different Age. The most common two buzzing bees that visit *S. violaceum* flowers were selected to study their handling time. Twenty flower buds ready to open were selected at the Kegalle site. The total time that a particular bee species buzzed at each of the selected flowers was recorded on 30/11/2009. On the following day, these flowers were considered 1-day old and the total handling time of each bee species at these anthers was recorded. On the third day, these flowers were considered 2-days old and the total handling time of each bee species was recorded. On the fourth day, these 20 flowers were considered >2-days old and the total handling time of each bee species at flowers were recorded. Accordingly, the total handling time of the two bee species on five new, 1-day-, 2-day- and >2-day-old flowers was observed only on sunny days (to minimize the effects from changing environmental conditions) from 30/11/2009 to 03/12/2009 for *H. westwoodi,* and from 04/12/2009 to 07/12/2009 for *P. kaluterae*. Ten specimens of each of the two common female (male bees do not buzz at flowers to collect pollen) buzzing bee species were measured for body length

to investigate the difference between the body length of the two buzzing bee species.

2.6. Study of the Efficiency of Buzz Pollination by Bees for Fruit Set and Seed Set of S. violaceum. The pollination trials were conducted from August 2009 to December 2009 in the site at Kegalle to study the efficiency of buzz pollination by bees for fruit set and the seed set of *S. violaceum*. Fifteen bunches of flower buds were randomly selected and covered by fine mesh bags to prevent visits of bees to flowers. Another randomly selected fifteen bunches of flower buds were tagged and kept open for bees to visit. Average number of fruits produced, and the number of seeds in each fruit in the two treatments were counted. Seeds obtained from each fruit from the two treatments were counted and allowed to germinate on wet tissues in Petri dishes. The number of germinated and ungerminated seeds produced from the two treatments (bagged and open) was counted.

2.7. Data Analysis. Data obtained from the study were analyzed using Minitab 14.0 and MS Excel-2007. MS Excel-2007 was used to compare the difference in the number of pollen grains in anthers of flowers at different age and the variation in handling time of the two common buzzing bee species at different age of flowers. A nonparametric test (Kruskal-Wallis test) was conducted to determine whether there is a significant relationship between the number of pollen grains per anther in flowers at different age. Two-sample t-test was carried out using Minitab 14 to determine whether there is a significant difference between handling time of the two common buzzing bees at flowers of each age category and the difference between the body length of the two common buzzing bee species at 95% confidence interval. The same analysis was conducted to test the difference in start of activity and end of activity between the non-buzzing bees and buzzing bees on *S. violaceum* flowers. The number of fruits, seeds per fruit, and the number of germinated seeds per fruit produced from the two treatments (open and bagged flowers) were also analyzed using two-sample t-test.

3. Results

In the nursery, seedlings appeared within 20 to 30 days after planting of seeds of *S. violaceum*. Seedlings took 15 days to reach the planting stage and 120–130 days to reach the reproductive stage. Upon maturity, the plants produced flower buds within five to eight days and they developed into flowers within one week. Fruits were produced within 65–70 days. On average, the plants took nearly seven months to produce mature fruits starting from the seedling stage. Flower buds took nearly one week to develop into flowers. New flowers opened around 7.30 a.m. and the lifespan of a flower was three to four days. Stigma of flowers remained receptive between 8.00 and 11.30 a.m. after blooming of flowers. Anthers dehisced between 7.30 and 8.00 a.m. Pollen grains of *S. violaceum* are yellowish-white, dry, and nonsticky with 0.02 mm of length and breadth.

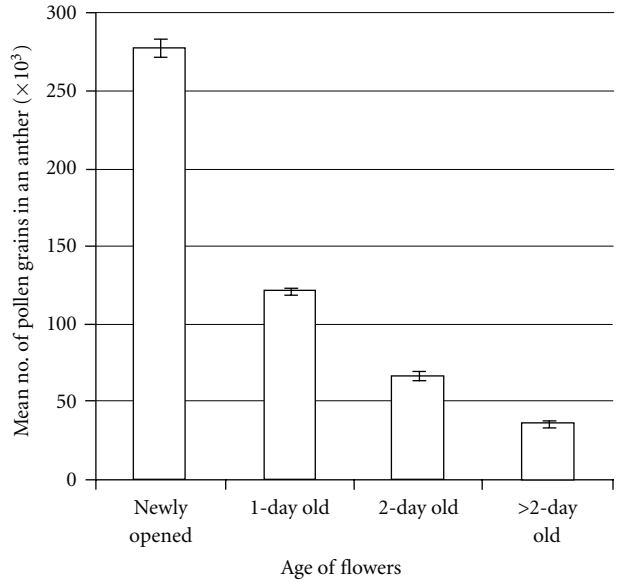

Figure 1: Variation in the mean number of pollen grains in an anther of *Solanum violaceum* as a function of the age of the flower.

3.1. Number of Pollen Grains in Anthers with Flower Age. Figure 1 shows the variation in the mean number of pollen grains in anthers with the age of the flower. The highest amount of pollen grains was found in new anthers. The lowest amount of pollen was found in anthers more than two days old. The results of the Kruskal-Wallis test were significant ($H = 17.97$, 3 d.f., $P = 0.000$) indicating that the mean number of pollen grains in anthers was significantly different among the different age categories of flowers.

3.2. Bees Visiting Flowers of S. violaceum. The preliminary survey conducted at Meewatura recorded four species of bees visiting flowers of *S. violaceum* of which, *Hoplonomia westwoodi* and *Patellapis kaluterae* (family Halictidae) were the buzzing bees and *Trigona iridipennis* and *Apis dorsata* (family Apidae) were the non-buzzing bees.

At the Kegalle site, *S. violaceum* flowers were visited by four species of buzzing bees namely; *Amegilla comberi*, *Xylocopa tenuiscapa* (family Apidae), *H. westwoodi* and *P. kaluterae* (family Halictidae), of which the latter two species were the most common. *Xylocopa tenuiscapa* was the rarest species and was mostly found hovering above the crop field. *Ceratina hieroglyphica*, *T. iridipennis* and *A. dorsata* (family Apidae) were the non-buzzing bees at this site. The non-buzzing bees were found collecting pollen spread over the flower petals that released due to the activities of the vibratile pollinators and they were found foraging on stigmae as well.

3.3. Activity Time of Pollen Carrying Bees on Flowers of S. violaceum. The pollen carrying bees were observed to study their activity time in flowers of *S. violaceum* on five sunny days. Activity time of buzzing and non-buzzing bees that visited *S. violaceum* flowers is given in Figure 3.

The first to visit flowers of *S. violaceum* were the buzzing bees and were followed by the honeybees. The starting time of activity between non-buzzing bees and buzzing bees was significantly different (P-value = 0.000, T-value = 4.79, DF = 25). However, the end of activity between non-buzzing bees and buzzing bees was not significantly different (P-value = 0.162, T-value = 1.45, DF = 23). The non-buzzing bees; *A. dorsata, T. iridipennis* and *C. hieroglyphica* were observed mostly after 9.30 a.m. The peak activity period during which most of the bee species were active on flowers in the Kegalle site was from 9.30 a.m. to 11.00 a.m.

3.4. Handling Time of the Two Common Buzzing Bee Species in Flowers at Different Age. *Hoplonomia westwoodi* (mean body length = 8.49 mm) and *P. kaluterae* (mean body length = 6.74 mm), of which the former bee is comparatively larger in size, were the most common buzzing bee species in the site at Kegalle. The body length of the two buzzing bee species were also significantly different (T-value = 54.44, P-value = 0.000, DF = 15). Figure 2 compares the mean handling time of the two buzzing bee species on newly opened, 1-day-old, 2-day-old and > 2-day-old flowers. The longest handling time of the two bee species was observed at new flowers while the shortest handling time was at flowers >2-days-old. There was a significant difference between the age of flower and handling time of *H. westwoodi* (H = 70.85, 3 d.f., P = 0.000) and *P. kaluterae* (H = 73.61, 3 d.f., P = 0.000).

There was a significant difference between the handling time of *H. westwoodi* and *P. kaluterae* on newly opened flowers (T-value = −7.38, P-value = 0.000, DF = 35), 1-day-old flowers (T-value = −5.23, P-value = 0.000, DF = 33), 2-day-old flowers (T-value = −4.83, P-value = 0.000, DF = 36) and more than 2-day-old flowers (T-value = −2.85, P-value = 0.009, DF = 35) at 95% confidence interval. Close observations revealed that, *P. kaluterae* vibrates each anther cone of a single flower separately, spending more time at a flower compared to *H. westwoodi* that vibrates all anther cones of a single flowers together at once.

3.5. Efficiency of Buzz Pollination in Fruit Set and Seed Set of S. violaceum

3.5.1. Fruit Set. Of the 15 flower bunches that contained about 230 flowers of *S. violaceum* kept open to facilitate bee visits, 95 fruits were formed representing 41.31% of the total flowers. The highest number of fruits (10 fruits) was obtained from one of the opened bunches which had 18 flowers (55.55%) while the lowest number of fruits (4 fruits) was obtained from the opened bunch which had 12 flowers (33.33%).

Of the other 15 flower bunches that contained about 230 flowers of *S. violaceum* kept closed by fine mesh bags to prevent bee visits, only 38 fruits were formed representing 16.52% of the total flowers. The highest number of fruits (4 fruits) was obtained from the closed bunch which had 18 flowers (22.22%) and lowest number of fruits (1 fruit) was from the closed bunch which had 14 flowers (7.14%).

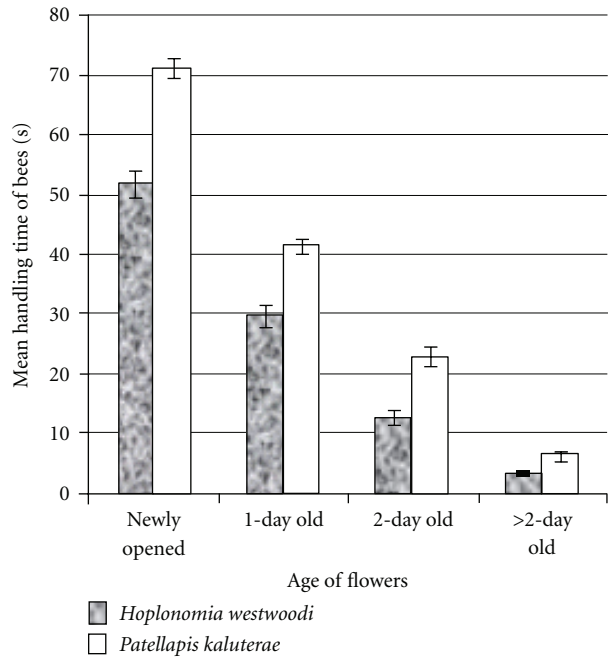

Figure 2: Mean handling time of *Hoplonomia westwoodi* and *Patellapis kaluterae* in *Solanum violaceum* flowers of different age in the site at Kegalle. (Mean temperature (°C), rain fall (mm), and relative humidity during the time of observations for the eight days: 30.11.2009—27.20°C, 0.0 mm, 90; 01.12.2009—26.8°C, 0.0 mm, 94; 02.12.2009—26.9°C, 0.0 mm, 88; 3.12.2009—27.0°C, 0.0 mm, 85; 04.12.2009—26.8°C, 0.00 mm, 78; 05.12.2009—26.4°C, 0.00 mm, 80; 06.12.2009—25.9°C, 0.0 mm, 79; 07.12.2009—26.1°C, 0.0 mm, 79).

Statistical analysis indicated that there is a significant difference between the number of fruits formed from open flowers and bagged flowers (T-value = −7.29, P-value = 0.000, DF = 22).

3.5.2. Seed Set. The two-Sample t-test for number of seeds formed from open flowers versus bagged flowers indicated that there is a significant difference between the number of seeds produced from open flowers and bagged flowers (T-value = 12.06; P-value = 0.000, DF = 108). Of the fruits formed from open flower bunches, 96% of the total number of 248 seeds were germinated. In fruits formed from bagged flower bunches, 92% of the total number of 208 seeds were germinated. Statistical analysis indicated that the number of germinated seeds produced from the open flowers were significantly different from that of bagged flowers (T-value = 6.34, P-value = 0.000, DF = 12).

4. Discussion

4.1. Bee Visitors of S. violaceum. As floral nectar is absent in flowers of *Solanum* [18], all the bees visited *S. violaceum* for pollen. The most common bees visiting *S. violaceum* flowers were buzzing bees belong to the family Halictidae that contains the highest number of bee species recorded for

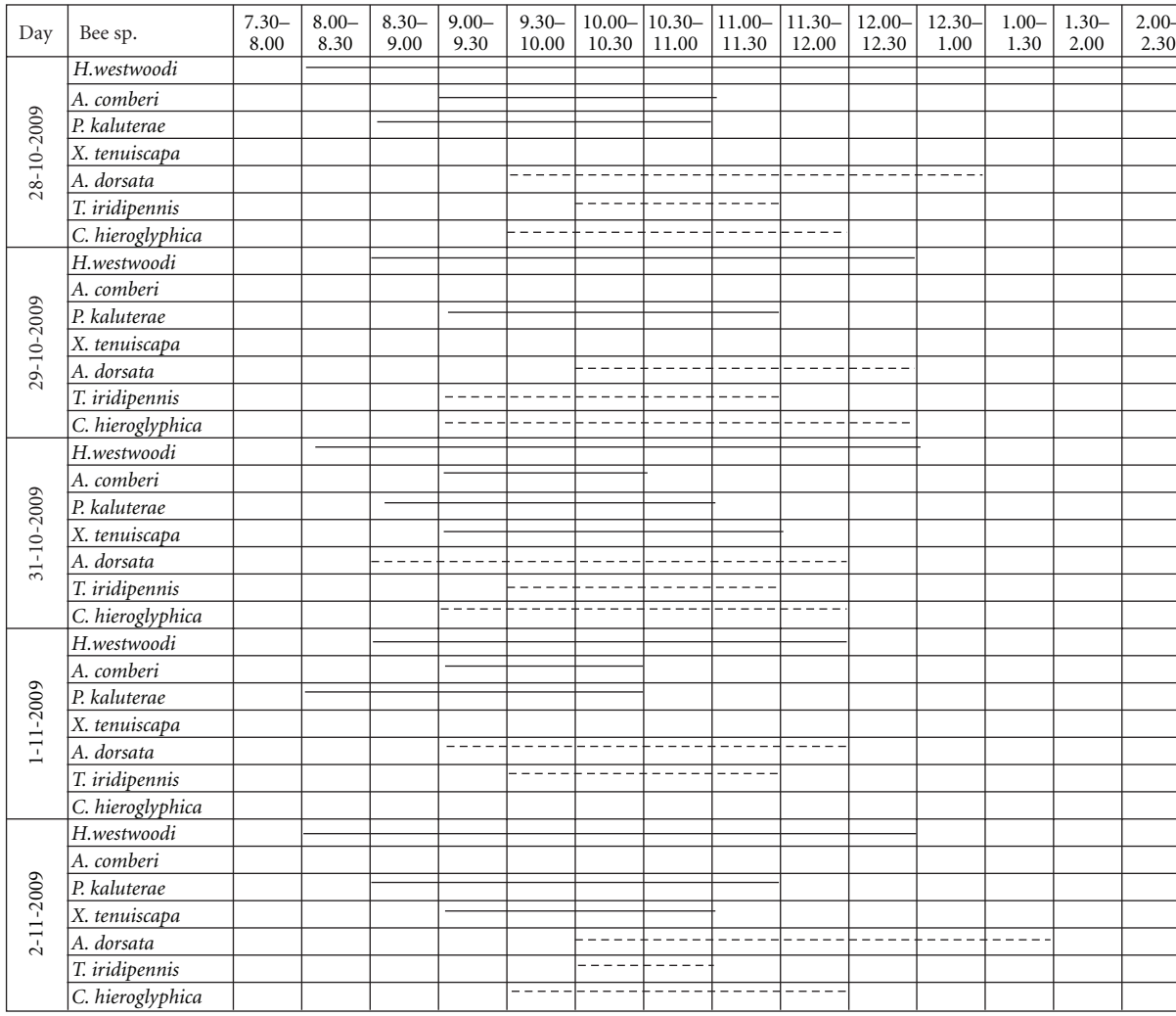

Figure 3: Activity time of seven species of bees (*Hoplonomia westwoodi*, *Amegilla comberi*, *Patellapis kaluterae*, *Xylocopa tenuiscapa*, *Apis dorsata*, *Trigona iridipennis*, and *Ceratina hieroglyphica*; — pollen bees, – – –honeybees) visiting flowers of *Solanum violaceum* from 7.30 a.m. to 2.30 p.m. during 5 sunny days in the site at Kegalle.

Sri Lanka [19]. The difference in the species composition of bees between the Kegalle site and the Kandy site indicates the site specificity of bee species visiting the same crop in different parts of the country. *Hoplonomia, Patellapis,* and *Amegilla* species are ground nesting bees [20] that cannot be reared by providing nesting places as for domesticated honeybees, leafcutter bees, and other stem nesting bees for crop pollination. This finding highlights the importance of conserving this wild bee fauna in an around crop fields even during the off season of crops. The three non-buzzing bees *Apis dorsata*, *Trigona iridipennis,* and *Ceratina hieroglyphica* may contribute to pollinate the tiny flowers of *S. violaceum* as they were found sometimes on stigmae of flowers. Anderson and Symon [15] report that *Trigona* species are very abundant on *Solanum* flowers with 99% floral fidelity and hence are significant pollinators. A similar study conducted in the Kandy site recorded *A. cerana*, the most common honeybee in Sri Lanka visiting flowers of *S. melongena* [21]. The absence of this species in *S. violaceum* flowers needs to be invesigated. An islandwide survey of insects visiting *S. violaceum* would document the different species of buzzing and non-buzzing bees in different parts of the country to reduce the biasness in results of the present study.

The buzzing bees observed during the present study are generalists that visit a wide range of flowers for pollen and nectar [10]. In flowers of *S. violaceum,* these generalist bees have become specialists to collect pollen, indicating that *S. violaceum* has restricted its pollen availability to a particular group of bees that can vibrate their anther cones to release pollen. The significant difference in the starting time of activity of buzzing and non-buzzing bees indicates the importance of the buzz pollinators to initiate pollen release that benefit the other non-buzzing bees visiting *S. violaceum*. Buzzing bees visited newly opened flowers more frequently than senescent ones with faded white petals and brown colour anthers [22, 23] and with no contrast that might provide the long-distance cue to identify the depletion

of pollen in anthers with flower age [11, 24, 25]. These signals may help the bees to spend their energy only for successful floral visits.

4.2. Age of Flower, Pollen Availability, and Handling Time of Buzz Pollinators. The release of large amounts of pollen during initial vibrations of new flowers by bees [26] decreases the amount of pollen with its age. The depletion in the number of pollen grains per anther with age of the flowers is correlated to the handling time [8, 11, 27, 28] of *H. westwoodi* and *P. kaluterae*. Harder and Barclay [12] suggested similar finding for *Solanum* flowers. The decreased handling time of the two buzzing bees, *H. westwoodi* and *P. kaluterae,* with the age of the flower might be due to the low availability of pollen which is evident from the age-dependant changes in flowers. Buchmann [5] stated an opposite finding to the present study, where the handling time is shorter at new flowers than at older flowers, as bees tend to buzz longer times in flowers with low pollen amounts. This might be possible in systems with low pollen availability, if the emerging of the buzz pollinators is seasonal and also to reduce competition among the buzzing bees active at the same time which is not similar to the present study system.

The body size of *H. westwoodi* and *P. kaluteare* inversely correlated with their handling time [15]. The difference in handling time between the two species is most likely due to their behavior in vibrating the anther cones, either singly or collectively depending on their body size. This finding is also supported by Buchmann [5] and Shelly and Villalobos [11]. According to Symon [9], only large insects are capable of buzzing and removing pollen from the anthers and function as pollen vectors. The two common bee species recorded during the present study are comparatively smaller bees. However, the largest bee in Sri Lanka, *X. tenuiscapa* that visited the flowers of *S. violaceum* that are smaller, could not handle the anther cones efficiently.

4.3. Buzz Pollination for Fruit Set and Seed Set in Solanum violaceum. The significant difference in fruit set and seed set between the two treatments may be mostly due to bee visits that facilitate removal and transport of pollen in open flowers. Buzzing bees visiting open flowers might have either cross-pollinated, self-pollinated, or might have pollinated by both methods efficiently than the closed flowers that might have self-pollinated in the absence of buzzing bees. The pollen from the cloud due to vibration by bees may directly land on stigma of the same flower facilitating self-pollination. If the bee has pollen on its body collected during previous visits from other plants, it may land on the stigma facilitating cross-pollination. Pollen from anthers of bagged flowers may release due to wind vibrations. Therefore, it seems that *S. violaceum* flowers are using both the biotic and abiotic pollinating agents for its pollination [29–32]. Close observations should be carried out to monitor bee visits to investigate the contribution of cross-pollination by bees to enhance seed set and fruit set versus self-pollination in *S. violaceum*. Kakizaki [33], Jones and Rosa [34] and Pal and Taller [35] report that *Solanum* plants grown in cages without bees produced no fruits. Baily [36] and Aizen et al. [37] report that a large number of seeds were produced by cross-pollination by bees compared to artificial pollination which resulted in fewer seeds in *S. melongena*. Fandino [24] reported that the mean number of seeds obtained from self-pollination was lower than from pollination experiments with bumblebees, showing that buzz pollination is more suitable for reaching a higher seed formation. The significant difference between the number of germinated seeds obtained per fruit produced from bagged flowers and open flowers may be due to bee pollination of open flowers that facilitates cross-pollination. In the presence of bees, germinability of *Brassica napus* has increased from 83% to 96% [38], which is also evident from the present study. The findings of the present study emphasize the importance of natural wild bees in pollinating the local naturalized exotic crops under natural conditions.

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Research Article

Pollination Biology of *Potentilla recta* (Sulfur Cinquefoil) and Its Cooccurring Native Congener *Potentilla gracilis* in Northeastern Oregon

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Pollination biology of the invasive plant sulfur cinquefoil (*Potentilla recta* L.) and it's native cooccurring congener slender cinquefoil (*P. gracilis* Dougl. ex. Hook.) was studied from 2002–2004, at four sites in northeastern Oregon, USA The native cinquefoil flowered first for five weeks, followed by the invasive for five weeks, with two weeks overlap in mid-June. Invasive flowers attracted 74 species and 543 individuals; the native attracted 93 species and 619 individuals. The most important pollinators for the invasive, in order of importance, were: *Apis mellifera*, *Ceratina nanula*, *Halictus tripartitus*, *Lasioglossum sisymbrii*, and *Bombus rufocinctus*; for the native: *C. nanula*, *Trichodes ornatus*, *H. ligatus*, *L. sisymbrii*, and *L. olympiae*. The invasive produced higher numbers of seeds per plant, having greater mass per unit vegetation. Mean seed size was lower for the invasive when pollinators were allowed access to flowers, but seed size increased linearly with more complete exclusion of pollinators; the native showed no such response to pollinator exclusion. Compared to the native, nearly twice as many seeds germinated for sulfur cinquefoil (35.0% versus 19.5%), with seeds germinating over a longer period of time. Results are discussed as they relate to the invasiveness of sulfur cinquefoil relative to the native.

1. Introduction

Nonnative invasive plants are increasingly recognized as major threats to ecosystems worldwide, particularly in arid and semiarid regions [1]. In western North America, invasive plants have changed fire regimes [2], reduced livestock forage quality, damaged real estate and recreation values [3], and impacted biodiversity [2]. While their influence on biodiversity has been described well in terms of the structure of native plant communities, relatively less is known on their ecological relationships to other species, including those that play critical functional roles, such as pollinators.

Insects, especially bees, beetles, flies, and butterflies are known to pollinate a majority of vascular plant species worldwide; beetles alone have been observed to pollinate 211,935 species, or over 88% of the total species of vascular plants [4]. Insects also play a major role in crop reproduction: Williams [5] estimated that 84% of crop species in the European Union are pollinated by insects, and Buchmann and Nabhan [4] reported that 67 principal crop species are pollinated by insects worldwide, out of 84 listed (80%). The key to effective pollination service is diversity, since most native insect pollinator species visit only a small set of potential flowering plant species [6], and since many plant species are designed to be pollinated by only a small set of available pollinators [7]. As a result, most ecosystems require a diversity of both plants and pollinators in order for effective pollination to be carried out [8].

When exotic plants invade native communities, plant species diversity can decline, and this may lead to concomitant decreases in the diversity of native pollinator communities. Furthermore, the spread of invasive plants, especially those that reproduce only by seed [9], may be dependent on how successful they are at competing for the service of resident pollinators. Thus pollinators can act to exacerbate the spread of invasive plants, by providing a service that improves seed production and the colonization potential of these species [10, 11]. Unfortunately, basic

information on the pollination ecology of invasive plants is lacking for most species. This information is especially critical for those species that reproduce primarily by seed, particularly if seed viability depends on outcrossing [11].

This study describes the pollination biology of the invasive plant sulfur cinquefoil (*Potentilla recta* L; Rosaceae) and its native cooccurring congener slender cinquefoil (*P. gracilis* Dougl. ex. Hook.), in northeastern Oregon. Sulfur cinquefoil is native to Eurasia and was introduced into North America before 1900 [12]. It is now naturalized across much of the United States and southern Canada, occurring from British Columbia east to Newfoundland and Nova Scotia, south to Florida, and west to eastern Texas [12–15].

In northeastern Oregon, sulfur cinquefoil occurs in open grasslands, shrubby areas, and disturbed areas including old fields, roadsides, pastures, and fencerows [16]. Sulfur cinquefoil can be highly competitive and has been observed to invade bluebunch wheatgrass (*Pseudoroegneria spicata*) rangeland in good condition and to displace other invasive species at some sites [17]. Sulfur cinquefoil is unpalatable to most livestock and wildlife, primarily because of its high-tannin content [12, 18, 19]. In fact, cattle will selectively graze spotted knapweed, another unpalatable species, in preference to sulfur cinquefoil [19]. As a consequence, overgrazing, which reduces competition from grass and other competing vegetation, generally favors sulfur cinquefoil [20].

Like its native congeners [21], sulfur cinquefoil is a long-lived perennial forb, having one to several erect, stout stems 30–70 cm tall growing from a woody caudex [12, 17, 18]. Peak flowering generally occurs in late June, depending on locality [12, 18]. Sulfur cinquefoil reproduces primarily by seed, and although self-fertilization can occur, most seeds are produced by cross-fertilization [22]. Seeds do not have a special dispersal mechanism [22]. Seeds germinate naturally at anytime during the growing season [23], and most vegetative growth occurs early the following spring [19].

In northeastern Oregon, sulfur cinquefoil cooccurs with its native congener *P. graclis* at many localities [24], and this presented the opportunity to study its pollination biology relative to the native. In particular, a comparative study of plants of both species living side by side could shed light on the extent to which the invasive has evolved distinct strategies to attract and retain pollinators, relative to the native congener. The current study compares the pollination biology of *P. recta* and *P. gracilis* by investigating respective flowering phenology, pollinator community structure, pollinator preference, nectar rewards, fidelity of pollen transfer, and influence of pollinator exclusion on seed set, seed size and number, and germination timing and rate.

2. Study Sites and Methods

The study was conducted between May 2002 and July 2004, in northeastern Oregon, where cinquefoil grows in small meadows intermixed with trees and shrubs (Figure 1). The general area experiences a Pacific Maritime Climate, warm and dry from late June to October, and cool and wet from November through May. Between 1965 and 2005, annual mean daily high temperature in La Grande, OR was 16°C, annual mean low temperature was 3°, and annual precipitation was 43.5 cm. Four study sites were selected for this study (Figure 1): the "Foothill" site (800 m elevation), just south of and closest to the largest municipality (La Grande OR, USA), was also the most dominated by sulfur cinquefoil (>95% *P. recta*); the "Rice" site was at slightly higher elevation (1000 m) on Glass Hill Road, 5 km southwest of La Grande, and here *P. recta* represented about 70% of total *Potentilla* cover; the "Ham" site (elevation 900 m) was located on Hamburger Hill, between Imbler and Elgin, 15 km northeast of La Grande, at which *P. recta* represented 50% of total *Potentilla* cover and the "Morgan" site was located at Morgan Lake, 10 km west of La Grande (1200 m elevation), and here *P. recta* represented just 10% of the total *Potentilla* cover.

In May 2002, we established five circular 400 m^2 plots (11 m radius) at each of the four study sites, within which most subsequent fieldwork was undertaken. Plots were selected so as to represent the approximate invasive to native composition of *Potentilla* species at that site. To determine flower phenology, relative flower availability was assessed at weekly intervals throughout each flowering season at each site, by counting the number of open flowers of each species (invasive or native) within each 400 m^2 plot. To determine the structure of the pollinator community of each cinquefoil species through time (species composition and relative abundance), we collected and identified all flower visitors within each plot at weekly intervals throughout each flowering season (2002, 2003, and 2004). By combining data on pollinator community structure for the two plant species within each plot with the relative abundance of flowers for each species, we could determine pollinator preference, by calculating an "electivity" index [25] for each flowering species, with the use of the following equation:

$$\mathrm{EI}_a = \frac{R_a - P_a}{R_a + P_a}, \tag{1}$$

where EI_a is Electivity index for plant species a, R_a is proportion of total pollinator population visiting plant species a, and P_a is proportion of plant species a in total flowering population.

This index will range between (−1), indicating total avoidance by pollinators of that plant species, to nearly (+1), which would indicate total dominance by that plant species of the pollinator resource. A value of (0) would indicate no preference for flowers of the given species of plant.

To gain insight into the potential efficacy of insects for distributing cinquefoil pollen, we removed and identified pollen from at least ten individuals of the 20 most common flower visitor species. Pollen was brushed off the bodies of randomly selected pinned individuals onto glass slides and preserved with standard methods. Pollen was identified with the use of a reference collection obtained by extracting pollen from flowers curated in the plant collection at Eastern Oregon University. In June and July 2003, we measured nectar quality in flowers of *P. recta* and *P. gracilis*, using a hand-held refractometer. Flowers of both species were collected, centrifuged, and a capillary tube was used to extract and measure the quality of nectar (% solute).

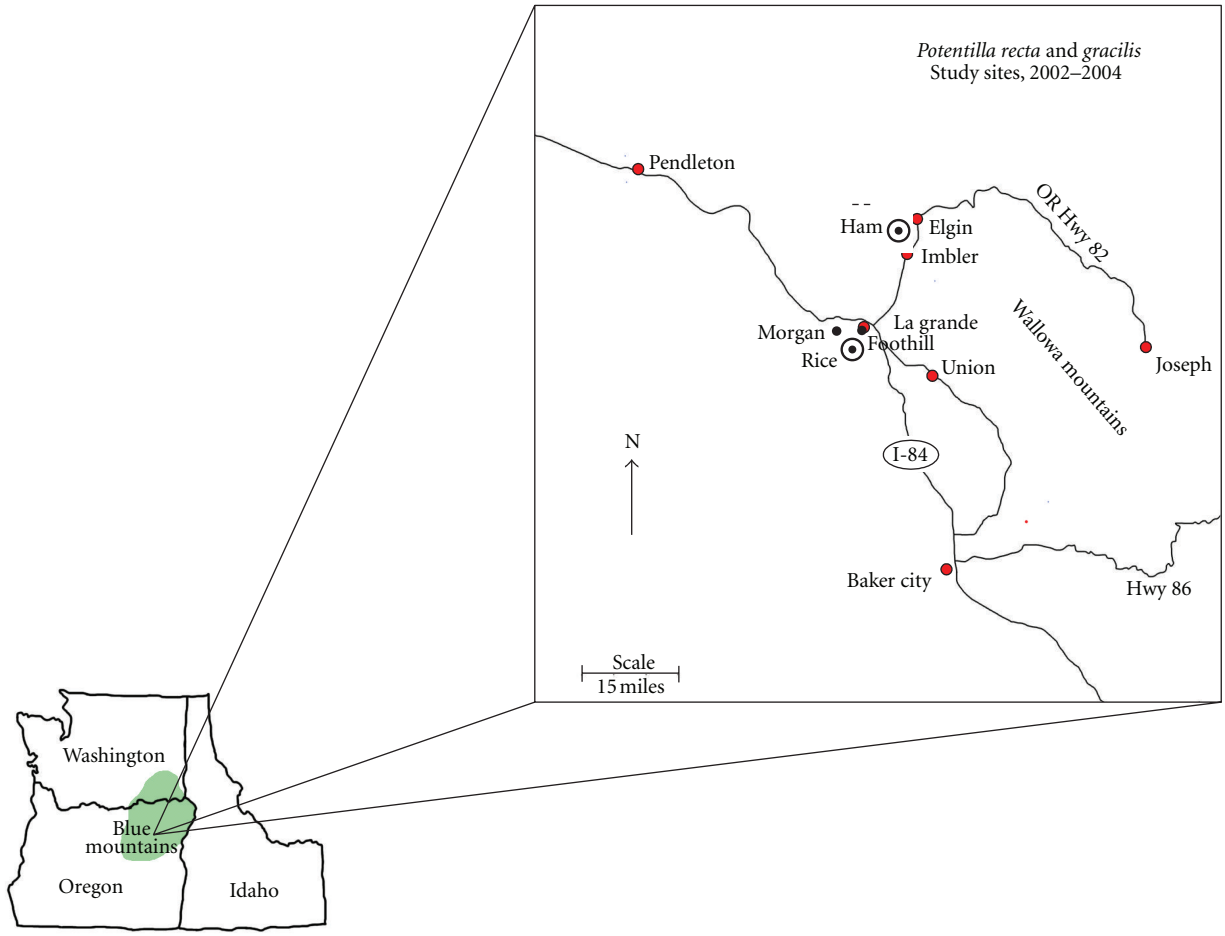

Figure 1: Map of *Potentilla* study sites, northeastern Oregon, 2002–2004.

Between late May and early July 2003, at two of the four study sites (Ham and Rice), we conducted a pollinator exclusion experiment designed to measure the potential influence of pollinators on seed set, seed size, and germination rate. Following the general protocol of Barthell et al. [11], five treatments were applied, four of which featured flower-head exclusion bags that varied in mesh size, designed to exclude pollinators of various sizes (Figure 2): (1) 1-mm mesh size: excluded all pollinators, regardless of size; (2) 3-mm mesh size: allowed access to the smallest pollinators, such as most Halictids and small Syrphid flies, but excluded medium and large pollinators such as most Apids, Megachilids, Andrenids, and large Syprhid and Bombyliid flies; (3) 5-mm mesh size: allowed access to small and medium-sized pollinators, but excluded the largest pollinators such as Bombids; (4) 10-mm mesh size: a "sham" cage, designed to test for bag effects *per se*: technically allowed access to all pollinators, regardless of size; and (5) no bag: flowerheads were left in the natural state, which allowed uninhibited access to all pollinators. One complete block of the five treatments was applied to a total of 240 flowerheads, 120 at each site, with each flowerhead representing an individual cinquefoil plant. At each site, we established four separate transects, separated by at least 100 m, along which we positioned 30 randomly selected plants, 15 of which were sulfur cinquefoil, alternating with 15 that were native cinquefoil. Bags were installed at least one week prior to flowering (late May to early June), and because flowerheads continued to expand during the experiment, bags had to be regularly re-positioned to accommodate new growth. Throughout the experiment, we visited bag installations on a weekly basis, to check for bag damage or other problems in installation, to record unexpected ingress of insects into the bags, and to count visitors on unbagged flowers of each species. Once flowering ceased, experimental bags were replaced with opaque cotton "seed bags," to insure that no seeds escaped from flowerheads as the seeds within them matured in the weeks following the cessation of flowering. After flowerheads had stopped growing and had clearly senesced (by the end of July), flowerheads were removed from plants and taken back to the lab for processing. In early August, flowerheads were oven-dried, dissected, and all seeds removed, counted, and weighed. To check for potential effects of seed predation on germination, a

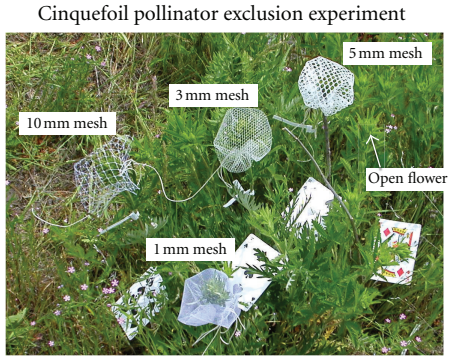

Figure 2: Photograph of a block of the five treatments deployed in the cinquefoil pollinator exclusion experiment: open flower (all access); 10 mm mesh size (sham cage, all access); 5 mm mesh (excludes large pollinators); 3 mm mesh (excludes large and medium pollinators); 1 mm mesh (excludes large, medium, and small pollinators).

total of 30 flowerheads of each species, 15 from both Rice and Ham sites, were dissected and checked for evidence of seed predation. To determine germination success over time, a subset of the total seeds (typically > 30) within each flowerhead were randomly selected, placed on moist filter paper within a covered petri dish, and monitored weekly for one year, and cumulative germination was determined.

Data on community structure are presented descriptively for all four study sites, as lists of species found through each of the three sampling seasons (2002, 2003, and 2004). The 20 most commonly collected pollinator species for each plant species are then compared descriptively. The ordination method "Nonmetric multi-dimension scaling" (NMS) [26] was used to characterize sites based on their composition and relative abundance of species, and then axes are correlated with site factors in an attempt to explain among-site and between-species patterns of community structure. NMS is ideal for ordination of most community data, because the technique is nonparametric, and thus does not assume any underlying distributional properties in the data set. Data on flower preference, using the electivity index were used to augment insights on the nature of patterns of pollinator use of the two plant species. For the bag experiment, we analyzed for the fixed effect of bag type on seed size, seed number per head, seed mass per head, and germination timing and rate with the use of a mixed general linear model, that included plant species, transect, and site as random factors.

3. Results

A total of 1,045 individual flower visitors were collected at the four sites over the three-year study period, comprising four orders, 36 families, and 111 species of insects (Table 7). Sulfur cinquefoil flowers attracted 74 species and 543 individuals, 16% of which were European honeybees (*A. mellifera* L.), while the native cinquefoil attracted a more diverse fauna of 93 species and 619 individuals, only 2% of which were honeybees. The 20 most commonly collected flower visitor species represented nearly 69% of the total individuals collected for each cinquefoil species (Table 1). Most pollinator species were "rare", reflected by the fact that 50 of 93 insect species observed on the native (53%) were represented by one or two individuals; for sulfur, 41 of 74 species were so represented (54%). Based on a combination of abundance and the presence of pollen on their bodies, the five most important pollinators for sulfur cinquefoil, in order of importance, were likely to be *A. mellifera, Ceratina nanula, Halictus tripartitus, Lasioglossum sisymbrii*, and *Bombus rufocinctus*; for the native, the most important pollinators were likely to be *C. nanula, Trichodes ornatus, H. ligatus, L. sisymbrii*, and *L. olympiae*. None of the 10 principle pollinators of each species were abundant throughout each respective flowering season, although for the native cinquefoil, most species were present throughout June, and for sulfur cinquefoil, most species were present from mid-June to mid-July (Table 2).

Although the pollinator fauna of the native cinquefoil was more abundant and rich than the fauna of sulfur cinquefoil, temporal (among-year) variance to mean ratios for pollinator abundance and richness for the native were roughly fourfold higher than for sulfur, and the spatial (among-site) variance to mean ratios were more than tenfold higher for the native, compared to sulfur (Table 3). Thus, it was much easier to predict both counts and species richness at any give time and place for sulfur cinquefoil, compared to the native. For example, despite equivalent sampling efforts at all sites each year, the native cinquefoil had very low abundance and richness of pollinators at the Foothill site, where sulfur cinquefoil dominated in percent cover (>90% sulfur), but very high abundance and richness of pollinators at the Morgan site, where the native dominated (90% native). In addition, the native pollinator fauna was roughly twice as abundant and three times as rich in 2003, as it was in the other two years (2002 and 2004). As a consequence, the invasive sulfur cinquefoil had a much more constant community of flower visitors over space and time compared to the native.

NMS ordination demonstrated few clear patterns of among-site, or between-cinquefoil species differences in pollinator communities. The most apparent pattern was the significant difference in community structure among survey years (Figure 3). The distinctiveness of the fauna in 2002 was represented best by Axis 2, with *C. nanula* (Apidae) and *L. sisymbrii* (Halictidae) having the highest correlations with Axis 2. The species that most indicated the position of the 2004 site samples, also correlated closely with Axis 2, were *Panurginus* sp. (Andrenidae) and *Coenonympha tullia* (Satyridae), followed by *Eristalis hirta* (Syrphidae), *Hylaeus episcopalis* (Hylaeidae), and *H. ligatus* (Halictidae). Axis 1 best separated 2003 as a distinctive year, and its strongest indicators were *E. hirta*, *L. olympiae*, *C. acantha*, and *H. farinosus* (Table 1).

Under field conditions of equal flower dominance, we were able to acquire preference data for nine taxa of pollinators (Table 4). Of these, only the European honeybee and two *Megachile* speices exhibited preference for sulfur cinquefoil (electivity index > 0), while two bee genera (five species) showed no preference (*Halictus, Bombus*), and 11 species in

Table 1: List of most commonly observed pollinator species (total abundance ≥ 5) for sulfur and native cinquefoils, ordered by abundance for each species, at four study sites in northeastern Oregon, 2002, 2003, and 2004. KEY to abbreviations: MO: Morgan Lake; FH: Foothill; RI: Rice; HH: Ham; NAT: Native Cinquefoil; SULF: Sulfur Cinquefoil.

Pollinator species	2002	2003	2004	MO	FH	RI	HH	TOT NAT
Panurginus sp. (UID)	0	27	68	11	0	15	69	95
Eristalis hirta	0	32	9	21	9	8	3	41
Ceratina nanula	40	1	0	0	0	2	39	41
Trichodes ornatus	16	6	10	2	0	23	7	32
Lasioglossum sp. (UID)	3	6	17	5	0	15	6	26
Lasioglossum olympiae	0	21	0	7	0	2	12	21
Halictus ligatus	7	8	5	5	0	1	14	20
Lasioglossum sisymbrii	17	0	0	3	0	2	12	17
Hylaeus episcopalis	0	11	4	7	0	7	1	15
Apis mellifera	0	11	3	1	5	0	8	14
Andrena sp. (UID)	0	11	3	8	1	5	0	14
Coenonympha tullia	0	5	8	1	0	5	7	13
Halictus tripartitus	1	11	0	4	0	1	7	12
Evylaeus sp. (UID)	0	11	0	9	0	0	2	11
Halictus sp. (UID)	1	7	2	0	0	2	8	10
Ceratina sp. (UID)	0	0	10	0	0	0	1	10
Colias sp. (UID)	7	0	0	0	0	0	7	7
Ceratina acantha	0	7	0	2	0	2	3	7
Bombus rufocinctus	0	7	0	3	1	2	1	7
Speyeria sp. (UID)	6	0	0	0	0	3	3	6
Osmia sp. (UID)	0	2	4	2	0	2	2	6
Halictus farinosus	1	5	0	2	2	2	0	6
Epicauta puncticolis	0	3	2	1	0	3	1	5
Total abundance	144	299	176	153	25	164	268	619
Total richness	48	133	55	56	13	51	54	93
Pollinator species	2002	2003	2004	MO	FH	RI	HH	TOT SULF
Apis mellifera	41	28	16	0	42	3	4	85
Lasioglossum sp. (UID)	19	1	18	4	6	2	8	38
Eristalis hirta	0	13	17	12	1	6	2	30
Ceratina nanula	24	1	0	5	8	3	9	25
Halictus tripartitus	15	5	5	9	5	7	4	25
Hylaeus episcopalis	0	9	12	9	0	7	5	21
Bombus bifarius	8	10	1	5	5	5	4	19
Lasioglossum sisymbrii	17	2	0	0	4	8	7	19
Ceratina sp. (UID)	0	7	10	9	0	0	8	17
Bombus rufocinctus	4	5	5	4	1	8	1	14
Halictus ligatus	1	1	12	1	7	3	3	14
Andrena prunorum	5	4	2	2	2	5	2	11
Panurginus sp. (UID)	0	2	8	4	5	0	1	10
Halictus sp. (UID)	1	0	8	0	6	1	2	9
Andrena sp. (UID)	0	3	5	4	0	4	0	8
Trichodes ornatus	6	0	1	1	0	5	1	7
Lasioglossum titusi	4	3	0	1	1	3	2	7
Megachile perihirta	2	2	3	2	1	2	2	7
Total abundance	222	164	157	107	144	127	102	543
Total richness	63	88	70	40	30	44	41	74

Table 2: Phenology of ten most commonly observed flower visitors of *Potentilla gracilis* (native) and *P. recta* (exotic), at four sites in Northeastern Oregon, 2002–2004.

Pollinator species	May 16–31	June 1–15	June 16–30	July 1–15	July 16–31
			P. gracilis		
Coenonympha tullia	46.2	38.5	15.4		
Trichodes ornatus	12.5	37.5	40.6	9.4	
Panurginus sp.	10.5	62.1	25.3	2.1	
Halictus ligatus	4.8	42.9	52.4		
Ceratina nanula		97.6		2.4	
Lasioglossum olympiae		85.7	14.3		
Lasioglossum sisymbrii		76.5	23.5		
Eristalis hirta		70.7	14.6	14.6	
Apis melifera		50.0	42.9	7.1	
Hylaeus episcopalis		6.7	66.7	26.7	
			P. recta		
Halictus ligatus		42.9	21.4	28.6	7.1
Bombus rufocinctus		7.1		92.9	
Bombus bifarius		5.3	47.4	47.4	
Eristalis hirta		3.3	76.7	20.0	
Apis melifera		1.2	64.7	32.9	1.2
Lasioglossum sisymbrii			42.1	57.9	
Hylaeus episcopalis			38.1	61.9	
Andrena pronorum			36.4	63.6	
Halictus tripartitus			12.0	64.0	24.0
Ceratina nanula				72.0	28.0

Table 3: Summary data for pollinator surveys for sulfur and native cinquefoil at four sites in northeastern Oregon, 2002, 2003, and 2004.

Sulfur cinquefoil			Native cinquefoil		
Year	Mean abundance per site	Mean richness per site	Year	Mean abundance per site	Mean richness per site
2002	55.5	15.8	2002	36.0	12.0
2003	41.0	22.0	2003	74.8	33.3
2004	39.3	17.5	2004	44.0	13.8
Mean/Year	45.3	18.4	Mean/Year	51.6	19.7
Var	79.6	10.4	Var	418.5	139.1
Var/Mean	1.8	0.6	Var/Mean	8.1	7.1
Site	Mean abundance per year	Mean richness per year	Site	Mean abundance per year	Mean richness per year
Morgan	107	40	Morgan	153	56
Foothill	144	30	Foothill	25	13
Rice	127	44	Rice	164	51
Ham	102	41	Ham	268	54
Total	543	74	Total	619	93
Mean/Site	120.0	38.8	Mean/Site	152.5	43.5
Var	372.7	36.9	Var	9909.7	417.7
Var/Mean	3.1	1.0	Var/Mean	65.0	9.6

5 taxonomic groups demonstrated preference for the native (*C. nanula*; *Andrena*—2 spp; *Hylaeus*—2 spp, Syrphidae—3 spp, Coleoptera—3 spp). These data roughly correspond to survey data, when pollinator species are ordered in terms of relative abundance for each of the cinquefoil species (Table 1).

Estimates of percent sugar concentration in nectar were more than six-fold higher for sulfur cinquefoil than for

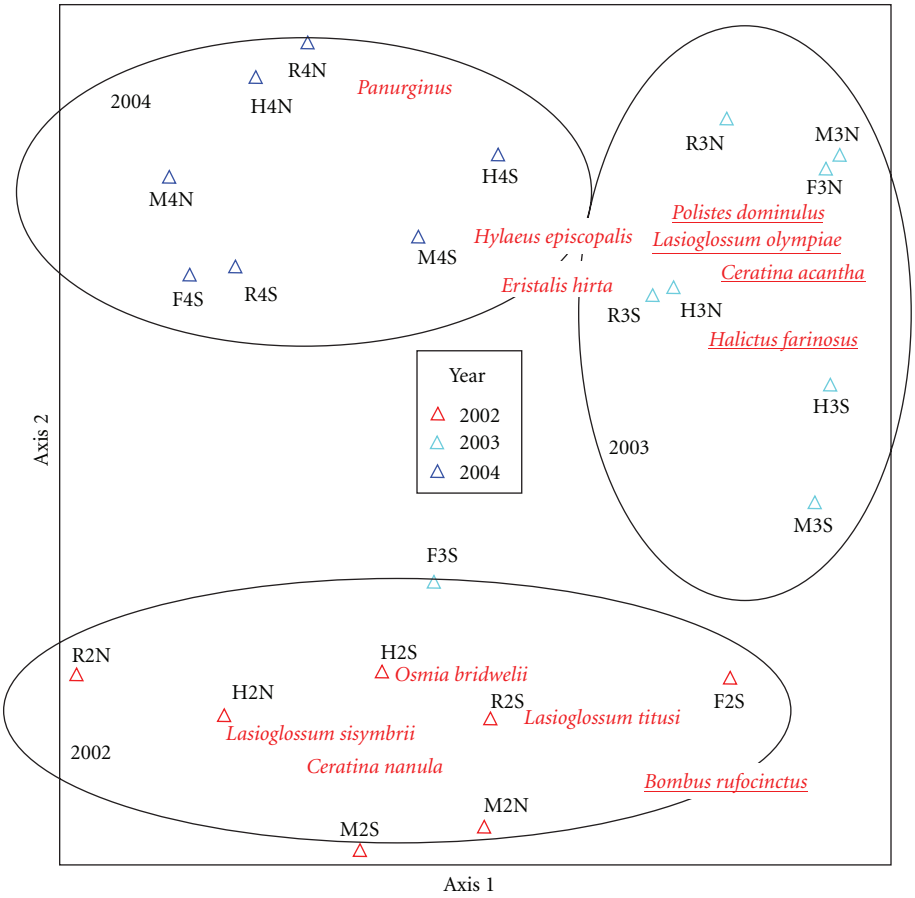

FIGURE 3: Ordination of sites by species for sulfur and slender cinquefoil pollinator communities at four sites in northeastern Oregon, 2002–2004. Key to sample acronyms (upright font): M = Morgan; F = Foothill; R = Rice; H = Ham; 2 = 2002; 3 = 2003; 4 = 2004; S = Sulfur; N = Native. Indicator species indicated in italicized font (Axis 2 correlates) and underlined font (Axis 1 correlates).

TABLE 4: Electivity indices for pollinator taxa under conditions of equal flower abundance. *R*: proportion of total pollinator population visiting plant sulfur cinquefoil; *P*: proportion of sulfur flowers among all cinquefoil flowers; *E*: electivity index = $(R_{\text{sulfur}} - P_{\text{sulfur}})/(R_{\text{sulfur}} + P_{\text{sulfur}})$.

Pollinator	Tot obs	No. of sulfur	No. of native	*R*	*P*	*E*
Apis mellifera	11	10	1	0.91	0.5	**0.29**
Megachile—2 spp.	5	4	1	0.80	0.5	**0.23**
Bombus—3 spp.	7	4	3	0.57	0.5	0.07
Halictus—3 spp.	21	9	12	0.43	0.5	−0.08
Andrena—2 spp.	13	3	10	0.23	0.5	**−0.37**
Ceratina nanula	19	4	15	0.21	0.5	**−0.41**
Hylaeus—2 spp.	5	1	4	0.20	0.5	**−0.43**
Syrphidae—3 spp.	6	1	5	0.17	0.5	**−0.50**
Coleoptera—3 spp.	10	0	10	0.00	0.5	**−1.00**

the native cinquefoil (59.0 ± 0.8 S.E. versus 9.6 ± 0.3 S.E.). These estimates correspond to observations indicating that honeybees were much more attracted to invasive flowers compared to the native.

When seed parameters are compared between the two species for the unmanipulated (open) treatment, several differences were observed. First, mean individual seed mass was significantly higher for the native compared to sulfur cinquefoil (0.207 mg mean seed mass ± 0.003 S.E. for the native, versus 0.172 mg ± 0.003 S.E. for sulfur), and these differences were consistent for both the Ham and Rice sites. Second, the native cinquefoil produced significantly fewer seeds per head than did sulfur (1202 seeds per head ± 103 S.E. for the native versus 1817 ± 104 S.E. for sulfur), although seed production by the native was significantly lower at the Rice site. Despite having significantly smaller

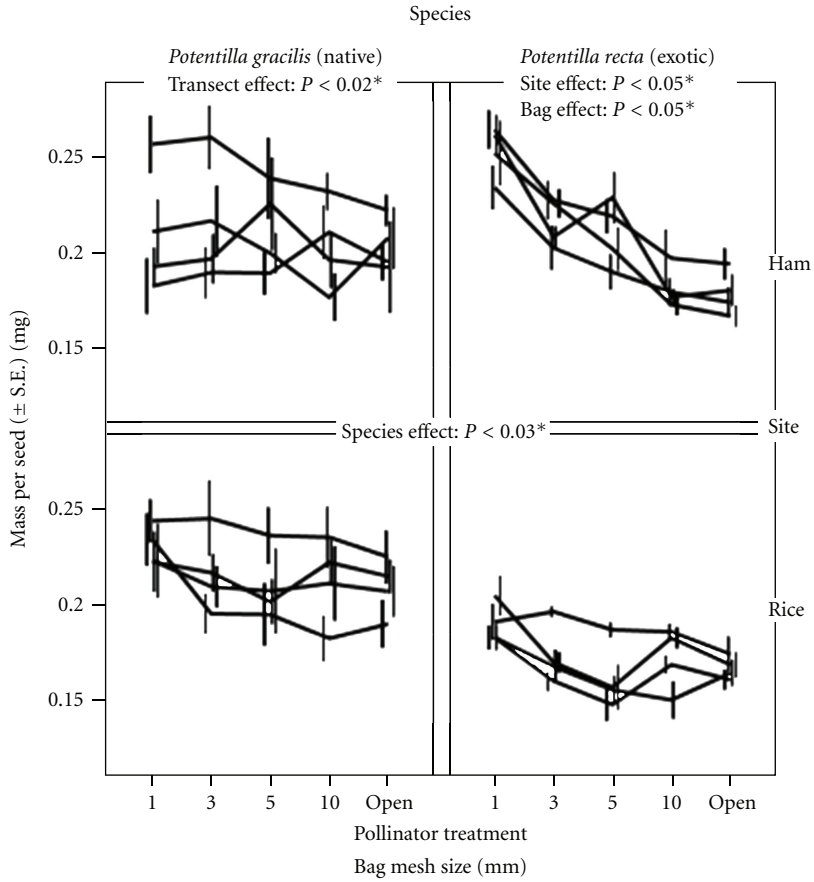

Figure 4: Mass per individual seed (mg) for *Potentilla gracilis* and *P. recta* seeds produced by flower heads exclosed by bags having mesh sizes designed to exclude various sizes of pollinators, along four transects at both Ham and Rice sites, northeastern Oregon, June-July 2003.

seeds, overall differences in seed number per head translated into significantly higher total seed mass per head for sulfur, compare to the native (0.31 g mass per head ± 0.02 S.E. for sulfur, versus 0.23 g ± 0.02 S.E. for the native). Once again however, native production was lower at the Rice site.

Pollinator exclusion at the Rice and Ham sites caused significant changes in seed parameters for both species of cinquefoils, but effects were much more pronounced for sulfur cinquefoil and were generally of greater magnitude at the Ham site. At both sites, sulfur cinquefoil plants produced progressively larger seeds as bag treatments became limiting to progressively smaller pollinators (Figure 4). This effect; however, was somewhat site-specific, with the Ham site exhibiting a more pronounced effect ($P < 0.0001$), compared to the Rice site ($P < 0.05$). This is reflected by the magnitude of increases in mean seed mass for sulfur cinquefoil between the open treatment and the most exclusive 1 mm bag treatment: at the Rice site, mean seed mass increased just 14% from 0.17 mg (± 0.003 S.E.) to 0.19 mg (± 0.004 S.E.), while at the Ham site, mean seed mass increased 41% from a mean of 0.18 mg (± 0.004 S.E.) to a mean of 0.25 mg (± 0.006 S.E.). For the native, mean seed mass did not generally increase with progressive decreases in bag mesh size, though at the Rice site, mean size mass increased slightly from 0.21 mg (± 0.004 S.E.) to 0.23 mg (± 0.008 S.E.). For the number of seeds per exclosed head, significant effects were observed only at the Ham site. For the native, although there was no significant bag effect overall for seed number, plants at the Ham site that had received the most exclusive bag treatment (1 mm) produced seed heads having significantly fewer seeds compared to the open treatment (928 seeds/head ± 85 S.E. versus 1538 ± 161 S.E.). For sulfur, the experimental results at the Ham site were also distinctively different than for the Rice site in terms of seed number per head. In particular, flower heads that were exclosed by the 1 mm bag produced only 1/2 of the total seeds per head compared to the open treatment (949 seeds/head ± 61 S.E. versus 1892 seeds/head ± 179 S.E.). In contrast, at the Rice site, plants of neither species responded significantly to treatments in terms of seed number per head. Overall however, for sulfur cinquefoil, the larger seeds observed in the 1 mm bag were produced at the expense of a significantly lower seed number per head, although this effect was much more pronounced at the Ham site. For total seed mass per head, effects were not obviously progressive when comparing all bag treatments, and also tended to vary with site, in much the same way as for the number of seeds per head. For the native, while there were no significant treatment effects on seed mass per head when all bag treatments were analyzed together, seed mass at the Ham site decreased significantly ($P < 0.01$) from 0.28 g per head (± 0.02 S.E.) in the open treatment to 0.18 g per head

(± 0.01 S.E.) for the 1 mm bag, representing a 36% decrease. At the Rice site; however, the decrease was only 9% and was not significant. For sulfur cinquefoil, once again seed mass per head decreased significantly only at the Ham site, from 0.33 g (± 0.03 S.E.) for the open treatment to 0.24 g (± 0.01 S.E.) for the 1 mm bag treatment, representing a 27% decrease; at the Rice site, both treatments produced a mean seed mass per head of 0.29 g. Finally, note that the among-site variation presented above was augmented by within-site variation, as reflected by the four transects located at each experimental site (Figure 4). In particular, note that variation among transects was substantial, both in mean seed mass, and in the pattern of response across treatments, especially for the native cinquefoil. Clearly, while pollinator exclusion had clear effects in some cases, the magnitude of spatial variation at two scales makes it risky to predict what might happen with a similar experiment at other sites.

There is no obvious explanation for the observed differences in treatment effects between the Rice and Ham sites. These two sites were similar in elevation, aspect, and general landscape conditions, and while seed productivity was much higher at Ham for the native, sulfur cinquefoil plants produced roughly similar seed numbers and seed mass at the two sites in the open condition. To assess whether the greater magnitude of effects at the Ham site could have been due to higher numbers of pollinators or a more diverse pollinator community there, we observed patterns of flower visitation during the experiment. These data indicate that site differences cannot be explained by either the number or community structure (Table 5) of pollinators that may have been excluded: the richness and species composition of pollinators observed at flowers of plants neighboring those that had received treatments were roughly similar for the Rice and Ham sites (Table 5), and there were actually more pollinators available at the Rice site compared to the Ham site, during the experiment. Moreover, if the more subtle effects of treatment at the Rice site was due to a lower level of pollinator service, we would expect that seed numbers and mass per head would be equally high for the open versus 1 mm bags, instead of equally low, as we observed. For example, at the Rice site, mean seed mass per head for the native in the open treatment was only 0.16 g (± 0.02 S.E.), compared to 0.28 g (± 0.02 S.E.) in the open condition at the Ham site. If differences in pollinator community structure or overall abundance were responsible for the lack of effect at Rice, then we would have expected both the open and 1 mm treatment to have seed mass equally high, and more similar to the Ham site. Clearly, some other factor or set of factors was responsible for the difference in treatment effects between the Rice and Ham sites.

When other aspects of seed biology for the two plant species was compared, there were three distinct differences observed. First, the proportion of buds within which evidence of seed predation was observed was 0.22 for the native, compared to just 0.01 for the invasive (Table 6). Second, the invasive *P. recta* invested proportionally greater resources in seed production compared to the native cinquefoil, with total seed mass per head three times that observed for the native (Figure 5). Third, less than 20% of native cinquefoil

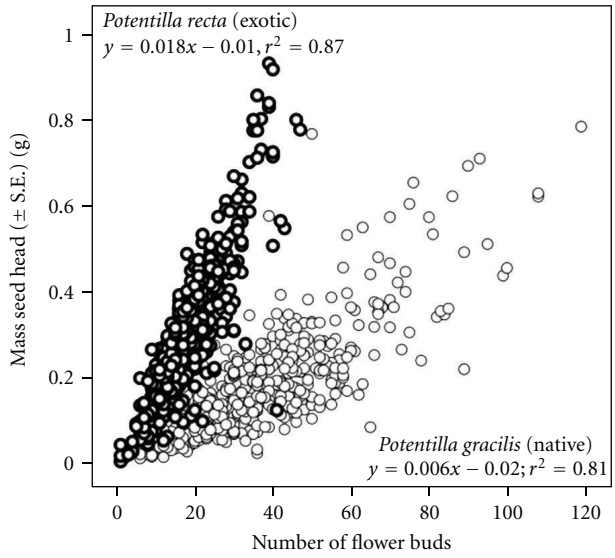

Figure 5: Mass of seed head (grams) as function of the number of buds in a head for *Potentilla gracilis* (native) and *P. recta* (invasive), at Rice and Ham sites, northeastern Oregon, June-July, 2003.

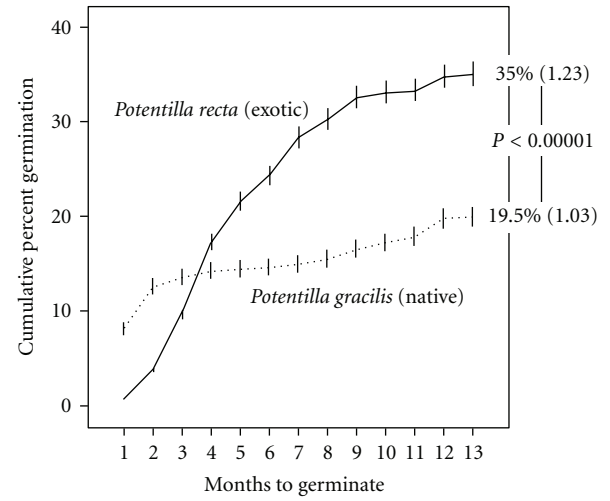

Figure 6: Cumulative percent germination by month for *Potentilla gracilis* (native) and *P. recta* (invasive), from seeds collected from plants at Ham and Rice sites, northeastern OR, July 2003–June 2004.

seeds germinated, and most germination occurred within two months after wetting, while 35% of sulfur cinquefoil seeds germinated, with germination occurring consistently for more than eight months after wetting (Figure 6). Finally, none of these germination parameters were significantly influenced by the pollinator exclusion treatment.

4. Discussion

Collectively, the flowers of cinquefoil attracted 111 insect species at four sites in northeast Oregon, but just 26 insect species comprised roughly 70% of total flower visitors observed. Although "pollinator quality" cannot be conclusively demonstrated in terms of plant fitness after Herrera [27], judging by the combination of relative abundance and

Table 5: List of pollinator species observed more than once, in order of abundance, for the 2003 flowering season at Ham and Rice sites, at *Potentilla recta* (exotic) and *Potentilla gracilis* (native) flowers, throughout the duration of the pollinator exclusion experiment conducted at these two sites, in June and early July 2003. Bold face refers to large bodied individuals, underline refers to medium bodied individuals, and light face refers to small bodied individuals.

Pollinator species	Rice native	Pollinator species	Ham native
Hylaeus episcopalis	6	*Panurginus sp.*	23
Lasioglossum sp.	5	*Lasioglossum olympiae*	12
Trichodes ornatus	4	*Halictus ligatus*	7
Eristalis hirta	4	*Halictus tripartitus*	7
Panurginus sp.	2	*Halictus sp.*	7
Lasioglossum olympiae	2	*Apis melifera*	6
Bombus rufocinctus	2	Bombyliidae 2	6
Andrena sp.	2	*Eristalis hirta*	3
Ceratina acantha	2	***Coenonympha tullia***	3
Andrena candida	2	*Ceratina acantha*	3
Bombyliidae UID	2	*Osmia sp. A*	3
Halictus farinosus	2	*Andrena augustitarsata*	2
Chlosyne paulla	2	***Aporinellus yucatanchsis***	2
Pollenia pseudorudis	2	*Lasioglossum (Evylaeus)*	2
		Melissodes bimatris	2
17 species Seen Once	17	20 Species seen once	20
Total richness	31	Total richness	35
Total abundance	56	Total abundance	108
Pollinator species	Rice sulfur	Pollinator species	Ham sulfur
Hylaeus episcopalis	6	*Apis melifera*	27
Eristalis hirta	6	*Hylaeus episcopalis*	3
Bombus rufocinctus	2	***Bombus bifarius***	3
Bombus bifarius	2	*Eristalis hirta*	2
Andrena sp.	2	*Andrena candida*	2
Andrena prunorum	2	*Colletes sp.*	2
Ceratina acantha	2	*Osmia pusilla*	2
Megachile perhirta	2	*Andrena thaspii*	2
14 species seen once	14	16 Species seen once	16
Total richness	22	Total richness	25
Total abundance	38	Total abundance	84

the presence of cinquefoil pollen on their bodies, perhaps just seven species performed most of the pollination service during the three-year study period. Although two abundant insect species served both species of flowers (the apid *C. nanula* and the halictid *L. sisymbrii*), the European honey bee was clearly the dominant pollinator in the mix, but only for the invasive sulfur cinquefoil. Moreover, the consistent dominance of the honey bee as the principle pollinator for sulfur cinquefoil was a primary factor explaining the much higher constancy of flower visitation by potential pollinators for sulfur cinquefoil than for its native congener.

Compared to most other studies, our collection of potential pollinators was very diverse. For example, we collected 60 species of bees over the three-year study period, compared to an average of just 19.6 (± 2.5 S.E.) species of bees in pollinator surveys of single species of plants [28]. Two of these studies are worth noting here. Richards [29] found a total of only 24 species (mostly *Megachile* and *Bombus* spp.) visiting cicer milkvetch (*Astragalus cicer* L.: Fabaceae) in southern Alberta, Canada, in a similar landscape setting, with a similar sampling effort, and over a similar time period (1978 to 1981). Richards and Edwards [30] found that just six species of bees (alfalfa leafcutting bee, honey bee, and four species of *Bombus*) served as pollinators of the forage legume sainfoin (*Onobrychis viciaefolia* Scop.) in southern Alberta from June to August 1986. Interestingly, sainfoin flower-handling time was inversely correlated with pollinator body size, with bumble bees able to extract nectar at a higher rate than honey bees or leafcutting bees, and thus it is possible that glossa length, which is also correlated with body size [31], might determine whether an individual bee can successfully extract nectar from zygomorphic flowers like legumes. However, nectar within simple, open flowers like cinquefoils, can be extracted by a wide variety of insect species, including not only bees, but flies, beetles, butterflies, and wasps. The only study we could find that reported a

Table 6: Proportion of cinquefoil buds ($N = 10$) within which evidence of insect activity was observed, for 15 paired samples of *Potentilla gracilis* and *P. recta*, at Ham and Rice sites, June, 2004.

Pair no.	Site	Species	Prop. buds infested	Type of insect activity	Species	Prop. buds infested	Type of insect activity
1	Ham	*P. gracilils*	0.1	Lepidoptera exuvia	*P. recta*	0	
1	Rice	*P. gracilils*	0.4	Diptera pupae	*P. recta*	0	
2	Ham	*P. gracilils*	0.0		*P. recta*	0	
2	Rice	*P. gracilils*	0.1	Diptera pupa	*P. recta*	0	
3	Ham	*P. gracilils*	0.1	Diptera pupa	*P. recta*	0	
3	Rice	*P. gracilils*	0.0		*P. recta*	0.1	Diptera pupa
4	Ham	*P. gracilils*	0.0		*P. recta*	0	
4	Rice	*P. gracilils*	0.2	Excrement	*P. recta*	0	
5	Ham	*P. gracilils*	0.1	Unknown insect parts	*P. recta*	0	
5	Rice	*P. gracilils*	0.2	Diptera pupae	*P. recta*	0	
6	Ham	*P. gracilils*	0.0		*P. recta*	0	
6	Rice	*P. gracilils*	0.1	Unknown insect parts	*P. recta*	0	
7	Ham	*P. gracilils*	0.0		*P. recta*	0	
7	Rice	*P. gracilils*	0.5	Unknown insect parts, Diptera pupae	*P. recta*	0	
8	Ham	*P. gracilils*	0.0		*P. recta*	0	
8	Rice	*P. gracilils*	0.4	Unknown insect parts, Diptera pupae	*P. recta*	0	
9	Ham	*P. gracilils*	0.1	Unknown insect parts	*P. recta*	0	
9	Rice	*P. gracilils*	0.7	Unknown insect parts, Diptera pupae	*P. recta*	0	
10	Ham	*P. gracilils*	0.2	Unknown insect parts	*P. recta*	0	
10	Rice	*P. gracilils*	0.6	Unknown insect parts, Diptera pupae	*P. recta*	0	
11	Ham	*P. gracilils*	0.2	Unknown insect parts	*P. recta*	0	
11	Rice	*P. gracilils*	0.4	Unknown insect parts, Diptera pupae	*P. recta*	0	
12	Ham	*P. gracilils*	0.0		*P. recta*	0	
12	Rice	*P. gracilils*	0.5	Unknown insect parts	*P. recta*	0	
13	Ham	*P. gracilils*	0.0		*P. recta*	0	
13	Rice	*P. gracilils*	0.6	Unknown insect parts, Diptera pupae	*P. recta*	0	
14	Ham	*P. gracilils*	0.0		*P. recta*	0.1	Diptera pupa
14	Rice	*P. gracilils*	0.7	Unknown insect parts, Diptera pupae	*P. recta*	0	
15	Ham	*P. gracilils*	0.1	Unknown insect parts	*P. recta*	0	
15	Rice	*P. gracilils*	0.2	Unknown insect parts, Diptera pupae	*P. recta*	0.1	Diptera pupa
Mean proportion		*P. gracilils*	0.22		*P. recta*	0.01	

more diverse pollinator fauna was our own study on the flower visitors of the invasive plant yellow starthistle (*Centaurea solstitialis* L.: Asteraceae), also conducted in northeast Oregon [32], over a similar time period (2000–2002). In that study, flowers of starthistle attracted 1923 individuals and an astonishing 203 species of insects, including 87 species of bees. Compared to the present study, this is 84% more individuals, 83% more total species, and 45% more bee species, observed with a similar sampling effort. The flowers of yellow starthistle are also relatively easy to access, and are also well known to produce copious quantities of rich nectar [33], so it is likely that the combination of rich nectar and easy access explains to a large extent the richness and abundance of the pollinator fauna of yellow starthistle.

It is interesting that between 2002 and 2004, the dominant pollinator of sulfur cinquefoil in northeastern Oregon was likely to be the European honey bee. This observation lends support to the idea that sulfur cinquefoil, like yellow starthistle [11], is part of an "invasive mutualism", in which the pollinator and the plant benefit from their relationship

Table 7: List of species observed visiting flowers of native (*Potentialla gracilis*) and sulfur (*Potentilla recta*) cinquefoil species at four sites in northeastern Oregon, 2002, 2003, and 2004. Site Codes: MO: Morgan Lake; FH: Foothill; RI: Rice; HH: Hamburger Hill.

Order/Family/Species	Native																	Sulfur															
	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT NAT	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT SULF
Coleoptera	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Buprestidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Cerambycidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Cerambycidae 1	0	2	0	0	0	0	0	0	0	0	0	1	2	0	0	1	**3**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Cerambycidae 2	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	0	0	0	0	0	1	1	1	0	2	0	0	0	3	2	**5**
Chrysomelidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Chrysochus cobaltinus	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Chrysomelidae 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	2	0	0	0	1	0	0	1	0	0	0	2	1	1	0	**4**
Cleridae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Trichodes ornatus	1	1	0	0	0	0	13	4	6	2	1	4	2	0	23	7	**32**	0	0	1	0	0	0	5	0	0	1	0	0	1	0	5	1	**7**
Meloidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Epicauta oregana	0	0	0	0	0	0	0	1	3	0	0	0	0	0	4	0	**4**	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0	**2**
Epicauta puncticolis	0	1	0	0	0	0	0	1	2	0	1	0	1	0	3	1	**5**	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	**2**
Lytta morenes	0	0	0	0	0	0	2	1	0	0	0	0	0	0	3	0	**3**	0	0	0	0	0	0	2	0	0	0	0	0	0	0	2	0	**2**
Coleoptera 1	0	1	0	0	0	0	0	2	5	0	0	0	1	0	7	0	**8**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Diptera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Asilidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Geron argatus	0	3	0	0	0	0	0	0	0	0	0	0	3	0	0	0	**3**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Bombyliidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Anastoechus barbatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	1	0	0	0	0	0	2	0	0	0	1	0	2	0	**3**
10 Unidentified Spp.	1	0	0	0	0	0	2	2	0	3	7	0	1	0	4	1	**15**	0	1	1	4	2	0	1	1	1	6	1	2	2	6	12	9	**29**
Calliphoridae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Pollenia pseudorudis	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Nemestrinidae	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Sarcophagidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Blaesoxipha sp.	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0	**2**	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0	**2**
Sarcophagidae 1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Syrphidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Chrysotoxum fasciatum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**
Eristalis hirta	0	17	4	0	8	1	0	4	4	0	3	0	21	9	8	3	**41**	0	0	12	0	5	5	0	6	0	0	2	0	12	1	6	2	**30**
Parasyrphus relictus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	**2**
Platycheirus obscurus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	**1**
Spaerophoria sulphuripes	0	1	0	0	1	0	0	1	0	0	0	0	1	1	1	0	**3**	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	2	**2**
Syrphus opinator	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0	**2**
6 Unidentified Spp.	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	**2**	0	2	0	17	4	0	2	0	0	0	1	0	2	21	2	1	**26**
Tabanidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
4 Unidentified Spp.	0	1	0	0	0	0	0	1	0	0	0	0	1	0	1	0	**2**	0	0	0	3	0	0	1	0	0	0	0	0	0	3	1	0	**4**

Table 7: Continued.

Order/Family/Species	Native																	Sulfur															
	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT NAT	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT SULF
Tachinidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Peleteria malleola	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	**1**
Tachinidae 1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Diptera 1	0	4	2	0	3	0	0	0	5	0	1	4	6	3	5	5	**19**	0	0	0	0	0	3	0	0	0	0	0	1	0	3	0	1	**4**
Heteroptera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Miridae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Irbisia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**
Hymenoptera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Andrenidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Andrena augustitarsata	0	2	0	0	0	0	0	0	0	0	2	0	2	0	0	2	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Andrena candida	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	**2**	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	**2**
Andrena miranda	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**
Andrena nigrocaerulea	0	3	0	0	0	0	0	1	0	0	0	0	3	0	1	0	**4**	0	0	0	0	0	0	0	1	1	0	0	0	0	0	2	0	**2**
Andrena pensilis	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Andrena prunorum	0	1	0	0	1	0	0	1	0	0	0	0	1	1	1	0	**3**	0	1	1	2	0	0	3	2	0	0	1	1	2	2	5	2	**11**
Andrena quintiliformis	0	1	0	0	0	0	0	1	0	0	0	0	1	0	1	0	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Andrena scurra	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	**1**	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	**2**
Andrena thaspii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	1	0	0	0	0	0	0	0	0	2	0	1	0	0	2	**0**
Andrena sp. 1	0	8	0	0	1	0	0	2	3	0	0	0	8	1	5	0	**14**	0	1	3	0	0	0	0	2	2	0	0	0	4	0	4	0	**8**
Andrena sp. 2	0	1	0	0	0	0	0	1	0	0	1	0	1	0	1	1	**3**	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**
Andrena sp. 3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Andrena sp. 4	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Panurginus sp.	0	2	9	0	0	0	0	2	13	0	23	46	11	0	15	69	**95**	0	2	2	0	0	5	0	0	0	0	0	1	4	5	0	1	**10**
Apidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Anthophora urbana	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	**2**	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	**2**
Apis melifera	0	0	1	0	5	0	0	0	0	0	6	2	1	5	0	8	**14**	0	0	0	29	0	13	2	1	0	1	27	3	0	42	3	4	**76**
Bombus bifarius	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	5	0	4	0	1	3	2	0	1	3	0	5	5	5	4	**19**
Bombus californicus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	1	0	0	0	0	1	0	0	0	0	1	0	1	0	**0**
Bombus huntii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	**1**
Bombus insularis	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	**2**
Bombus nevadensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	3	0	0	1	0	0	0	0	0	0	3	1	0	**4**
Bombus rufocinctus	0	3	0	0	1	0	0	2	0	0	1	0	3	1	2	1	**7**	0	2	2	1	0	0	3	2	3	0	1	0	4	1	8	1	**14**
Ceratina acantha	0	2	0	0	0	0	0	2	0	0	3	0	2	0	2	3	**7**	0	1	0	0	0	0	0	2	0	0	1	0	1	0	2	1	**4**
Ceratina nanula	0	0	0	0	0	0	1	1	0	39	0	0	0	0	2	39	**41**	4	1	0	8	0	0	3	0	0	9	0	0	5	8	3	9	**25**
Ceratina sequoiae	3	0	0	0	0	0	1	0	0	0	0	0	3	0	1	0	**4**	2	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	**2**
Ceratina sp. 1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	**1**	0	7	2	0	0	0	0	0	0	0	0	8	9	0	0	8	**17**
Melissodes bimatris	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Melissodes lutulenta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	**5**
Melissodes sp. 1	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	**2**	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	**1**

Table 7: Continued.

Order/Family/Species	Native																	Sulfur															
	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT NAT	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT SULF
Chrysididae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Chrysididae 1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Colletidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Colletes gypscolenes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	**1**
Colletes simulans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	**2**
Colletes sp. 1	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	**2**	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	**2**
Hylaeinae	2	2	0	0	0	0	0	0	0	0	0	0	4	0	0	0	**4**	1	0	0	4	0	0	5	0	0	0	0	0	1	4	5	0	**10**
Hylaeus episcopalis	0	5	2	0	0	0	0	6	1	0	0	1	7	0	7	1	**15**	0	0	9	0	0	0	0	6	1	0	3	2	9	0	7	5	**21**
Hylaeus nunemacheri	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Crabronidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Crabronidae 1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Eumenidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Eumenidae 1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	**2**
Halictidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Agapostemon virescens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	**2**
Halictus farinosus	1	1	0	0	2	0	0	2	0	0	0	0	2	2	2	0	**6**	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	**2**
Halictus ligatus	0	1	4	0	0	0	0	0	1	7	7	0	5	0	1	14	**20**	0	0	1	0	0	7	0	0	3	1	1	1	1	7	3	3	**14**
Halictus sp.	0	0	0	0	0	0	0	0	2	1	7	0	0	0	2	8	**10**	0	0	0	0	0	6	1	0	0	0	0	2	0	6	1	2	**9**
Halictus tripartitus	1	3	0	0	0	0	0	1	0	0	7	0	4	0	1	7	**0**	2	3	4	5	0	0	5	1	1	3	1	0	9	5	7	4	**0**
Lasioglossum olympiae	0	7	0	0	0	0	0	2	0	0	12	0	7	0	2	12	**0**	0	2	0	0	0	0	0	1	0	0	0	0	2	0	1	0	**4**
Lasioglossum sisymbrii	3	0	0	0	0	0	2	0	0	12	0	0	3	0	2	12	**0**	0	0	0	2	2	0	8	0	0	7	0	0	0	4	8	7	**0**
Lasioglossum titusi	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**32**	1	0	0	0	1	0	2	1	0	1	1	0	1	1	3	2	**7**
Lasioglossum (Dialictus)	0	1	0	0	0	0	0	1	0	0	1	0	1	0	1	1	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Lasioglossum (Evylaeus)	0	9	0	0	0	0	0	0	0	0	2	0	9	0	0	2	**4**	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**2**
Lasioglossum sp. 1	0	1	4	0	0	0	3	5	7	0	0	6	5	0	15	6	**8**	1	0	3	4	1	1	9	0	11	5	0	3	4	6	2	8	**0**
Sphecodes sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Ichnuemonidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Ichnuemonid 1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Megachillidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Hoplitis sp.	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	**2**	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**
Hoplitis albifrons	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Megachile brevis	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Megachile melanophaea	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Megachile perihirta	0	1	1	0	0	0	0	0	0	0	0	0	2	0	0	0	**2**	0	0	2	1	0	0	0	2	0	1	0	1	2	1	2	2	**7**
Megachile sp. 1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**
Osmia atrocyanea	0	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Osmia bridwelii	1	0	0	0	0	0	0	0	0	1	1	0	1	0	0	2	**0**	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	**0**
Osmia coloradensis	0	0	0	0	0	0	2	0	0	0	0	0	0	0	2	0	**2**	0	0	0	1	0	0	3	0	0	0	0	0	0	1	3	0	**4**
Osmia marginipennis	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Osmia pusilla	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	**2**
Osmia (Chenosmia)	0	2	0	0	0	0	0	0	1	0	0	1	2	0	1	1	**4**	0	0	2	0	0	0	0	0	0	0	0	0	2	0	0	0	**2**

Table 7: Continued.

Order/Family/Species	Native																		Sulfur															
	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT NAT		MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT SULF
Osmia sp. 1	0	0	2	0	0	0	0	1	1	0	1	1	2	0	2	2	**6**		0	0	2	0	0	0	0	1	0	0	0	1	2	0	1	1	**4**
Osmia sp. 2	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	3	**3**		0	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	**2**
Pompilidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Aporinellus yucatanchsis	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	**2**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Sphecidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Sphex pensylvanicus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**		0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	**7**
Sphecidae sp. 1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Vespidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Polistes dominulus	0	1	0	0	1	0	0	0	0	0	0	0	1	1	0	0	**2**		0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**
Stenodynerus canus	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Vespula germanica	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	**2**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Vespula pennsylvanica	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**		0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	**1**
Hymenoptera 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**		0	0	1	0	0	0	0	0	1	0	0	1	1	0	1	1	**3**
Hymenoptera 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**		0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	**1**
Lepidoptera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Hesperiidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Icaricia acmon	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Lycaenidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Plebejus saepiolus	2	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	**2**		2	1	0	0	0	0	0	0	0	0	0	0	3	0	0	0	**3**
Lycaenidae 1	3	0	0	0	0	0	8	0	0	2	0	1	3	0	8	3	**14**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Nymphalidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Chlosyne paulla	1	0	0	0	0	0	0	2	0	0	0	0	1	0	2	0	**3**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Speyeria sp.	0	0	0	0	0	0	3	0	0	3	0	0	0	0	3	3	**6**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Pieridae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Colias sp.	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	7	**7**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Satyridae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Coenonympha tullia	0	1	0	0	0	0	0	1	4	0	3	4	1	0	5	7	**13**		0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	**2**
Lepidoptera 1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Lepidoptera 2	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	**2**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**

in an exotic location. For sulfur cinquefoil however, it is clear that a host of native insect species offer pollination service, and thus contribute to its success as an invading species. In particular, even though honeybees dominated the pollinator fauna of sulfur cinquefoil, more than 80% of flower visiting individuals were native, including more than 70 native insect species, and 46 native species of bees. Overall, the importance of native pollinators to sulfur cinquefoil indicate that this invasive is well-integrated into the ecosystem of northeastern Oregon. Moreover, although populations of sulfur cinquefoil flower for only about 45% as much time as do populations of yellow starthistle [32], this invasive cinquefoil, like starthistle, is likely to play an important role in the life histories of at least some native insect flower-visiting species.

Pollinator community constancy, reflected by temporal and spatial variance to mean ratios, was much higher for sulfur cinquefoil than for its native congener. Much of the temporal and spatial variation in flower visitors of the native cinquefoil was due to highly variable counts of some of the common bee species that frequented the native, particularly species of the smaller-bodied apid genera *Ceratina* and *Panurginus* and species of the halictid genus *Lasioglossum*. It is widely known through longer term monitoring work, that bee species such as these typically experience wide fluctuations in abundance from year to year, and from site to site within years [34, 35]. Williams et al. [28] highlighted data from several studies demonstrating that the number of "singletons" (just one observation of a species in a given study), coupled with the magnitude of spatial and temporal variation in native bee count data at the species level is typically so high that sampling efforts must be very robust to capture meaningful shifts in actual population numbers over time. However, this does not explain why sulfur cinquefoil did not tend to be serviced by so many highly variable native species during the study period, but rather tended to attract species belonging to populations that experienced much less temporal and spatial variation. In any case, this observation suggests that sulfur cinquefoil attracts a very stable pollinator fauna where it occurs in northeastern Oregon and did not seem to be limited by pollination service at any site or at any time during the study period.

Our evidence suggests that while most native insect species do not prefer sulfur cinquefoil relative to its native congener, the invasive may be a partner in an "invasive mutualism", together with the European honey bee. The honey bee was by far the most common insect observed at flowers of sulfur cinquefoil during the study period, and clearly preferred the invasive when flowers of the two cinquefoils were of equal abundance. These data are supported by the work of Barthell et al. [11], working with yellow starthistle, in which the honey bee has been implicated as an important partner in the establishment and spread of that invasive in California. Although sulfur cinquefoil can clearly reproduce by selfing (unlike yellow starthistle), the distinct response of plants to pollinator exclusion suggests that there may be a fitness consequence of selfing. In any case, this relationship of sulfur to the honey bee, and the fact that native bees, flies, and beetles did not clearly prefer sulfur cinquefoil, but visited it in accordance with its relative abundance, is consistent with observations in other systems [36, 37]. In terms of mechanisms that may explain our data on preference, it is possible that the higher sugar concentration of nectar in sulfur cinquefoil served as an attractant to honey bees. However, other qualities of nectar that we did not measure, including the ratio of sucrose to hexose [38, 39], and the presence of key amino acids [40] may be attractants as well, and may be more important for explaining why native pollinators in northeastern Oregon do not generally prefer sulfur over its native congener.

Pollinator exclusion produced a greater response in seed parameters in the invasive sulfur cinquefoil, compared to the native slender cinquefoil. The most pronounced effect was that mean seed size increased with increasingly aggressive exclusion of pollinators, at the expense of a lower seed number as pollinator exclusion became more pronounced. This supports the finding of Werner and Soule [12], who worked on the biology of sulfur cinquefoil in Michigan. However, while mean seed mass under exclosed conditions increased by only 30% in our study (two sites combined), mean mass increased by 60% in the Michigan study. The difference between the studies was even more pronounced with seed number: in northeastern Oregon, flowers produced 68% as many seeds as did open flowers, compared to just 13% for the study by Werner and Soule [12]. It seems that the kind of variation observed within and between sites in northeastern Oregon is also present when this species is studied at other geographically distant sites. Actually, variation of this kind may be more the rule than the exception, as other studies have reported similar variation and inferred its adaptive significance. For example, Kasagi and Kudo [41] reported substantial temporal variation in self-compatibility in *Phyllodoce aleutica* (Ericaceae), with high self-compatibility corresponding with periods of pollinator limitation. Werner and Soule [12] did not discuss whether the production of larger seeds had any adaptive significance for sulfur cinquefoil, or whether seed size increase is merely a consequence of a change in the rate of seed production, induced by the lack of pollen at a critical time in development. In any case, we observed no difference in germination rate for the larger seeds produced in the bagged treatment. Additional research on the fate of fertilized seedlings, versus those produced by selfing, would be needed to establish the conditions under which selfing might be advantageous.

Compared to pollinator exclusion studies on more self-incompatible plant species (e.g., yellow starthistle; [11]), the magnitude of our results were subtle. Yellow starthistle responded to pollinator exclusion by producing very few seeds in the exclosed condition, lending support to the idea that pollinators such as honey bees are indeed "invasive mutualists" and tend to facilitate invasion of some exotic species. While it is clear that sulfur cinquefoil can produce viable seeds without fertilization, it is interesting nonetheless that this invasive species is markedly more responsive to pollinator exclusion than is its native congener.

In terms of seed biology, we observed three differences between sulfur cinquefoil and its native congener: for sulfur, the general lack of evidence of seed predation, the greater

allocation to seed production relative to vegetative biomass, and a much more prolonged germination sequence, lasting nearly a year. First, one of the best documented observations on invasive species is the lack of effective natural enemies in the first decades of invasion [42]. Our observations on seed predation support the idea that native seed predators have not had sufficient time to adapt to the smaller sulfur cinquefoil seeds since introduction occurred a little more than 100 years ago. Indeed, although we did not establish a causal connection between the magnitude of seed predation and germination rates, it is noteworthy that proportionally nearly twice as many sulfur seeds germinated as did the native. Second, sulfur cinquefoil dedicated three times as much energy to reproduction each year during the three-year study period as did the native cinquefoil. It has long been observed that ruderal plant species tend to allocate proportionally greater resources to reproduction, even under relatively stressful environmental conditions [43, 44]. This strategy seems to balance the increased risk of mortality in the parent, with the increased opportunity for survival of the offspring. Similarly, the much longer germination "window" observed in sulfur cinquefoil, relative to the native, may be a strategy for retaining opportunity to take advantage of disturbed habitats over a longer period of time. Sulfur cinquefoil is highly successful at "filling in" suitable habitat once it arrives on the scene [45]. A longer germination window may be one mechanism this invasive species uses to gradually occupy an area once it colonizes. The native cinquefoil species on the other hand, can only respond to disturbance in a previously colonized area within a short period of time each year (~2 months), and thus may be at a competitive disadvantage over the long run, where it cooccurs with sulfur cinquefoil.

In general, our comparative data indicate that the invasive sulfur cinquefoil and the native slender cinquefoil employ different adaptive strategies, with the invasive using more of a "ruderal" strategy, as opposed to a "stress-tolerant" strategy used by the native [44]. Sulfur cinquefoil is clearly preferred as a nectar source by honey bees, utilizes a suite of native pollinators as well, invests relatively more energy in seed production, and enhances its chances to seize opportunities for disturbed conditions over a much longer period of time relative to the native cinquefoil. While our observations underline key differences in life history between sulfur cinquefoil and its native congener, additional work is required to understand exactly how these differences may translate into fitness differentials in the long run.

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Research Article

Effects of Long Distance Transportation on Honey Bee Physiology

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Despite the requirement of long distance transportation of honey bees used for pollination, we understand little how transportation affects honey bees. Three trials in three different states (CA, GA, and MI) were conducted to study the effects of long distance transportation on honey bee physiology. Newly emerged bees from one colony were split into two groups and introduced into a transported (T) colony or a stationary (S) colony in each trial. Volumes of hypopharyngeal gland acini in T colonies were significantly smaller than S colonies in all three trials. There were no significant differences between S and T colonies in juvenile hormone titers. Protein content in head showed no significant differences between S and T either in 7-day-old or 17-day-old bees of MI trial, but GA trial showed a significant reduction in bees experiencing transportation. Protein content in thorax was only measured in GA trial and was not significantly different between the two groups. Lipid content in abdomen was not significantly different between the S and T colonies in all three trials. This study suggests that bees experiencing transportation have trouble fully developing their food glands and this might affect their ability to nurse the next generation of workers.

1. Introduction

Honey bees (*Apis mellifera*) are perhaps the most important insects to humans due to their pollination service provided to agriculture [1]. Honey bees experience many different types of stresses. They are impacted by parasitic mites such as *Varroa destructor* and *Acarapis woodi* [2], microsporidian pathogens such as *Nosema apis* and *Nosema ceranae* [3], hundreds of pesticides applied in crops and brought back by foragers [4], as well as pesticides beekeepers applied inside the colonies [5]. Besides these stresses, perhaps the strongest stress experienced by honey bees is long distance transportation. For example, bees are transported from Florida to California, across four time zones in the spring for almond pollination. About 50% of bee colonies in Michigan also migrate to south (e.g., Georgia and Florida) for overwintering and then are moved back for apple and cherry pollination. Yet we understand little of the effects of the long distance transportation on bees because no studies have ever been conducted to determine the physiological or behavioral changes induced by such stress.

Honey bees have an age-related division of labor whereby young workers stay inside taking care of brood (nurses), and old bees forage for food (nectar and pollen) and other resources (water and propolis). This progression of behavioral changes is associated with juvenile hormone (JH), with nurses having low levels of JH and foragers having high ones [6–8]. Although JH is not considered a stress hormone [9], JH titers in blood can tell us whether workers are switching to become foragers or not. JH has been shown to be antagonistic to vitellogenin (Vg) [10], whereby bees with low JH always have high Vg and high JH suppresses Vg. Vg has been shown to be associated with slower aging because it protects workers from oxidative stress [11, 12] and is higher in queens [13], who live longer than workers. Workers that are ready to forage are characterized with high JH, low Vg, low abdomen fat, and lower body protein, essentially becoming "disposable" from the colony's point of view. JH therefore is a reliable indicator of the physiological age of workers. The hypopharyngeal glands (HPGs) of honey bees play a critical role in social

cohesion, because they provide secretions rich in protein, which are fed to larvae of all three castes, and also to adult queen, drones [14, 15], and foragers [16]. The sizes of HPG reflect how good a protein nutrition the bees have obtained prior to becoming nurses and may affect their nursing ability [17, 18] or it can reflect the effect of pathogens such as *Nosema apis* [19]. The amount of abdomen fat is another indicator for when workers become foragers, with nurses having high levels and foragers low levels [20]. Protein levels in workers will tell us whether workers during transportation can still obtain adequate protein nutrition or not. For example, can they still find/eat the same amount of pollen while "on the move" on the truck? Are their digestion efficiency affected by transportation?

In this study, we determined for the first time whether bees undergoing long distance transportation have higher JH levels (aging prematurely) and whether their hypopharyngeal gland sizes, total protein content in head or thorax, and lipid content in abdomen are smaller or lower due to transportation. Our hypothesis was that either due to higher mortality of older bees during transportation or loss of them due to drifting after transportation, or due to inadequate pollen consumption by young bees, we should see higher JH titers, smaller HPGs, lower protein content in heads and thorax, and lower lipid content in abdomen, in the bees experiencing transportation.

2. Materials and Methods

2.1. General Methods. A group of "transported" (T) honey bee colonies were moved to another location (CA trial), or traveled a round trip and returned to the original location (GA, MI trials). A group of "stationary" (S) colonies were not moved and served as the control. About 8–16 days prior to the transportation of bees, newly emerged bees from strong colonies were obtained by incubating sealed brood in make-shift "incubators" (34 ± 2°C, 50% RH, CA and GA trial), or a laboratory incubator (34 ± 1°C, MI trial). The incubating area was in a bathroom with 1-2 space heaters and a thermostat controlled power strip that powered the heater(s). Workers were obtained from a source colony (300–704 per colony), painted with Testor's color paint and split equally into two subgroups. One subgroup was introduced into a T colony, and the other subgroup was introduced into an S colony. Each of the S and T colonies receiving the same group of workers was termed a "colony-pair." This controlled any possible genetic differences among workers [21]. Any differences in measured parameters would be due to the treatment regime (transported or not), because colony differences (amount of food and brood) were controlled by having each colony-pairs as similar as possible in each trial. In each trial, for each total number of colony-pairs ($N = 6$ to 12), we obtained brood from $N + 3$ colonies to insure there were enough newly emerged bees for each colony-pair and did not use bees from the three lowest yielded colonies. Each colony pair therefore received bees from a single source colony.

Bee Bleeding. Hemolymph was taken for JH titer measurement. Sampled workers were bled according to established procedures [22]. Briefly, a small hole was pricked with a bent insect pin between the 4th and 5th abdomen segment of sampled bees. The hemolymph was collected in a capillary tube (Drummond Wiretrol 1 to 5 μL, Drummond Scientific company, USA) and then measured to the nearest 0.5 mm with a ruler and mixed with 500 μL of acetonitrile (EM Science) in a 12 × 125 mm culture tube with a Teflon-lined lid. The length of hemolymph was then converted to volume by knowing the calibrated mark (5 μL) as 27 mm long.

Juvenile Hormone Titer Measurement. Juvenile hormone (JH) was extracted from the hemolymph and assayed with established procedures [9]. Briefly, JH III in the hemolymph sample was extracted with 1 mL hexane (twice), then the pooled hexane was evaporated using a vacuum centrifuge (Speed Vac Plus SC110; Savant Instrument Inc., Holbrook, NY) linked to a condenser (Savant SS21), which trapped the solvent at −98°C. The dried JH in the sample tube was dissolved in 100 μL of methanol, and a 20 μL aliquot (in duplicate) was taken out, dried, and mixed with 200 μL of gel-phosphate-buffered-saline-Tritone (pH 7.3) containing anti-JH antiserum (1 : 14,000 dilution, generous gift from David Borst) and about 10,000 DPM of [10-3H(N)]-JH (Perkin Elmer, 647.5 Gbq/mmol). The mixture was incubated at room temperature for 2 h, and then 0.5 mL of dextran-coated charcoal suspension (Sigma) was added to each sample tube to absorb the unbound JH. This mixture was incubated in an ice-water mixture for 2.5 min and then centrifuged (2000 g for 3 min). The supernatant, which contained bound JH, was decanted into a glass scintillation vial. Liquid scintillation counting was performed using a Packard 2100TR. A standard curve with various amounts (0, 3, 10, 30, 100, 300, 1,000, 3,000, and 10,000 pg) of standard JH (Sigma) was run each day. KaleidaGraph (Synergy Software, PA, USA) was used to generate a standard curve. Five parameters for the standard curve were obtained by using DPM bound as the dependent variable, JH amount (after log transformation) as the independent variable, using nonlinear regression. The five-parameter formula was described in [22]. Excel (Microsoft, USA) was used to calculate the amount of JH in each sample, by reversing the five-parameter formula (solving for JH with known DPM and the five fitted parameters).

Hypopharyngeal Gland Size Measurement. Hypopharyngeal glands were dissected in 0.9% saline under a dissecting microscope (Olympus SZ12, x32) and then photographed by a digital camera (QImaging Go-3). We then used Image-Pro express 6.0 (Nikon, USA) to measure the width and length of five acini for each bee. The volume of each acinus was calculated as 1/6 × 3.14 × length × width [23].

Protein Content in Head and Thorax Measurement. Protein content in head and thorax was measured similar to hemolymph protein [24, 25]. Briefly, the head or thorax (excluding wings and legs) was removed with a pair of

micro-scissor from individual bees and crushed in 500 μL 1 N NaOH using a plastic pestle and incubated overnight. The solution was then vortexed and centrifuged for 5 min at 2000 g. The solution was then diluted 25 times and 10 μL was loaded (in duplicate) to a cell in a 96-cell plate, each cell was then added 200 μL Bio-Rad Protein dye (Bio-Rad, USA) after 4X dilution. The absorbance of the sample was measured at 595 nm using a Molecular Devices Softmax Pro5 Microplate Spectrophotometer. The amount of protein in each sample was calculated by comparing to a standard curve run each day using known amounts of bovine serum albumin (Sigma-Aldrich, USA).

Lipid Content in Abdomen Measurement. Lipid content in abdomen was measured similar to Toth and Robinson [20], using a colorimetric assay. Briefly, the abdomen was cut from individual bees and the internal organs (digestive tract and the sting apparatus) removed, leaving the cuticle with adhered fat body tissue. Each sample was then homogenized in a mechanical homogenizer (Polytron PT 2100, setting of 12) in 2 mL 2 : 1 chloroform : methanol [26] and allowed to extract overnight. The extract was then mixed with 0.5 mL water and centrifuged at 5000 g for 10 min. The top phase (water) was removed and discarded. The organic phase was filtered through glass wool and adjusted to a total volume of 2 mL. A 100 μL subsample of each lipid extract was dried completely, 0.2 mL concentrated sulfuric acid was added, and samples were heated in boiling water for 10 min. Then, 2.0 mL vanillin reagent (0.6% in 85% phosphoric acid) was added to each sample, which was vortexed and dark-incubated for 15 min to allow pink color formation. Absorbance at 525 nm was measured for each sample using a Molecular Devices Spectra Max 190 multiwell spectrophotometer (Sunnyville, CA, USA). A standard curve using known amounts of pure cholesterol was used to calculate lipid amounts. Each lipid sample was measured in duplicates, and average values were used for subsequent analysis.

2.2. Details of Three Trials

California (CA) Trial. Twelve colony-pairs were used; the S and T groups at Bakersfield, CA.

Newly emerged bees from source colonies were obtained and painted on March 12-13th 2008 then equally divided into two groups. One group was introduced into a T colony and the other half into an S colony. Over 6,000 bees were painted and introduced in two days (150 to 344 bees per colony, 24 colonies). The S group stayed in Bakersfield, CA, while the T group was moved to Florida during a 4 day period (March 14–17th) with a total distance traveled as 4,000 km. On March 18th, 6-7-day-old marked workers were sampled with soft forceps by two people (one at FL, another at CA), and placed them on dry ice, stored at -80°C, then shipped to Michigan State University (East Lansing, MI) for analysis.

Ten bees were thawed on ice, and blood removed for JH determination for each colony of 11 colony-pairs ($10 \times 11 \times 2 = 220$ bees) because one colony was lost due to robbing. A previous study has indicated that blood obtained this way showed lower JH titers compared to that of fresh bees, but the differences between nurses and foragers were maintained (Z. Y. Huang and K. Ahn, unpublished data). Ten bees were dissected for each colony of 9 colony-pairs for HPG size measurement ($10 \times 9 \times 2 = 180$ bees). Ten bees were measured in each colony of 4 colony-pairs for lipid content in abdomen analysis ($10 \times 4 \times 2 = 80$ samples).

Georgia (GA) Trial. Twelve colony-pairs were used for a second trial in Boston, GA. Nearly 6,000 bees (200 to 352 bees per colony) were painted and introduced into 24 colonies on April 18th and 19th, 2008 (but only the first cohort of bees, marked a different color, was used for sampling). The T colonies were moved to Sunfield, Michigan (07:00 April 20th to 15:00 April 21st), rested for one day (April 22nd), and then returned to Boston, GA (12:00 April 23rd to 16:30 April 24th), with a round trip of 3,250 km. The bees had opportunity to fly and forage on April 22nd, 2008, while in Michigan.

On April 25th, ten bees (8 days old) were sampled with soft forceps, put on dry ice for hypothermic anesthesia, and bled immediately (within 30 min) in each colony of the 12 colony-pairs for JH determination ($10 \times 12 \times 2 = 240$ bees). The bled bees were then individually labeled and frozen on dry ice, brought to Michigan, then stored at -80°C freezer until analysis. Since some heads were used to protein measurement, we only analyzed bees from 4 colony-pairs ($10 \times 4 \times 2 = 80$ samples) for HPG. Ten bees were analyzed in each colony of 8 colony-pairs for protein content in head and thorax ($10 \times 8 \times 2 = 160$ heads and 160 thoraces). Ten bees were analyzed in each colony of 4 colony-pairs for lipid content in abdomen ($10 \times 4 \times 2 = 80$ samples).

Michigan (MI) Trial. Six colony-pairs were used for S and T groups in East Lansing, MI. Newly emerged workers (192 to 336 bees per colony) were introduced to the colonies after being painted on May 19th of 2008 (1,430 bees) and 29th of 2008 (2,400 bees). These two groups were intended to be sampled as 17- and 7-day-old bees, respectively, on the date of sampling (June 4th). Transportation was conducted by driving the T colonies about 900 km per day (approximately 08:00 to 17:00), with a total of 2,750 km round trip (from June 1st to June 3rd).

On June 4th, paint-marked 7- and 17-day-old bees were collected by using soft forceps, bled, and stored at -80°C JH titers, HPG size, protein, and lipid analysis.

Ten 7- and 17-day-old bees were sampled in every colony of the 6 colony-pairs for JH determination ($10 \times 6 \times 2 = 240$ bees each age). Ten bees were dissected for HPG size measurement from each colony of the 6 colony-pairs ($5 \times 10 \times 6 \times 2 = 120$ samples). Ten bees were sampled in every colony of 5 colony-pairs for protein content in head analysis ($10 \times 5 \times 2 = 100$ heads). Ten bees were measured in each colony of the 6 colony-pairs for lipid content in abdomen ($10 \times 6 \times 2 = 120$ abdomens).

2.3. Statistical Analyses. Juvenile hormone titers were transformed (logarithmic (JH + 1)) to meet the requirements

of parametric analysis. Differences in JH titers, HPG size, total protein content in head or thorax, and abdomen lipid for bees in S and T groups were analyzed by ANOVA by State View (SAS Institute, NC, USA). Each colony-pair was analyzed separately as an independent comparison, but all colonies in each trial were also analyzed together to compare the overall effect of transportation.

3. Results

3.1. JH Titers in Hemolymph

CA Trial. There were no significant differences between the S and T groups, when all 11 colony-pairs were analyzed together by ANOVA (for F and P values, see Table 1), although colony-pairs 2 and 10 showed differences in JH titers when analyzed as two separate single colony-pairs (Figure 1(a)).

GA Trial. There were no significant differences between the S and T groups when all 12 colony-pairs were analyzed together (Table 1), although S showed significantly higher JH titers than T colony in colony-pair 8 (Figure 1(b)).

MI Trial. There were no significant differences between the S and T groups in 7-day-old bees when all 6 colony-pairs were analyzed together (Table 1), although colony-pairs 3, 4, and 6 showed significant differences between the two groups (Figure 1(c)).

In 17-day-old bees, no significant differences were detected between S and T groups when all 6 colony-pairs were analyzed together by ANOVA (Table 1), although the T had significantly higher JH titers than S colony in colony-pair 1 (Figure 1(d)).

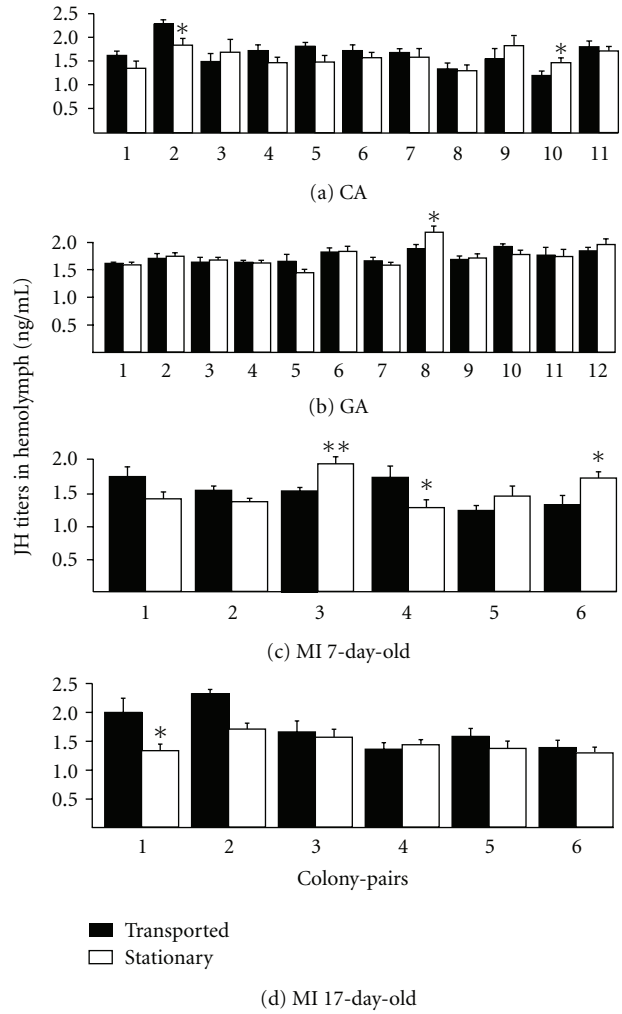

Figure 1: Hemolymph juvenile hormone titers (mean ± SE) in worker honey bees experiencing transportation (solid) or no transportation (open) in California (a), Georgia (b), or Michigan ((c), (d)). Workers were 6-7 days old for CA, 8 days for GA and noted in figure for MI. $N = 10$ bees for each colony. Each number represents a colony-pair that hosted genetically similar workers from a source colony. A* inside the open bar indicates that the JH titers between the two colonies within that colony-pair were significantly different (t-test, $P < 0.05$), while ∗∗ denotes highly significant ($P < 0.01$).

3.2. Volume of HPG Acini

CA Trial. There were significant differences in the volume of HPG acini between the S and T groups in the overall analysis (Table 1). If it was analyzed in each colony-pair, six pairs (except colony-pairs 3, 5, and 7) showed significant differences between the S and T colonies in the volume of HPG acini (Figure 2(a)).

GA Trial. Either by overall analysis (Table 1) or each colony-pair (Figure 2(b)), results showed that the volumes of HPG acini in the S group were larger than the T group.

MI Trial. When all colony-pairs were analyzed together by ANOVA, results showed that the volumes of HPG acini were significantly different between the S and T groups (Table 1) in 7-day-old bees. Although in colony-pair 5 the difference was reversed (Figure 2(c)).

In 17-day-old bees, the volume of HPG acini in S groups was significantly larger than T (Table 1), although there were no significantly differences in colony-pair 4, 5, and 6 (Figure 2(d)).

3.3. Protein Content in Head

GA Trial. When all 8 colony-pairs were analyzed together, ANOVA detected a significant reduction in head protein in the T group (Table 1), although only three colony-pairs (1, 3, and 8) showed that S groups had significantly higher protein content in heads when analyzed as single colony-pairs (Figure 3(a)).

MI Trial. For 7-day-old bees, when either analyzed together (Table 1) or as single colony-pairs (Figure 3(b)), there were

Table 1: *F* and *P* values from analysis of variance (ANOVA) conducted for each trial and each parameter, with "ns" denoting nonsignificant ($P > 0.05$). JH titers were transformed (log (JH + 1)) before ANOVA. "Colony source" refers to the differences due to genetic background of source colonies, "transportation" refers to the difference between transported and stationary colonies, and interaction refers to the effect of two (genetics X transportation status). Workers in CA were 6-7 days old, in GA were 8 days old, and those in MI were either 7 or 17 days old.

Item	Trial	Effect	DF	*F*	*P*
JH titers in hemolymph	CA	Colony source	10, 186	5.55	<**0.01**
		Transportation	1, 186	1.70	ns
		Interaction	10, 186	1.77	ns
	GA	Colony source	11, 214	5.98	<**0.01**
		Transportation	1, 214	0.01	ns
		Interaction	11, 214	1.24	ns
	MI 7-day-old	Colony source	5, 106	2.48	**0.04**
		Transportation	1, 106	0.02	ns
		Interaction	5, 106	5.31	<**0.01**
	MI 17-day-old	Colony source	5, 99	2.54	**0.03**
		Transportation	1, 99	6.08	**0.02**
		Interaction	5, 99	1.82	ns
Volume of HPG acini	CA	Colony source	8, 162	6.65	<**0.01**
		Transportation	1, 162	62.65	<**0.01**
		Interaction	8, 162	1.84	ns
	GA	Colony source	3, 72	0.52	ns
		Transportation	1, 72	43.39	<**0.01**
		Interaction	3, 72	0.23	ns
	MI 7-day-old	Colony source	5, 108	8.03	<**0.01**
		Transportation	1, 108	35.44	<**0.01**
		Interaction	5, 108	5.93	<**0.01**
	MI 17-day-old	Colony source	5, 108	5.11	<**0.01**
		Transportation	1, 108	18.17	<**0.01**
		Interaction	5, 108	4.16	<**0.01**
Protein content in head	GA	Colony source	7, 144	17.06	<**0.01**
		Transportation	1, 144	12.96	<**0.01**
		Interaction	7, 144	1.89	ns
	MI 7-day-old	Colony source	4, 90	12.27	<**0.01**
		Transportation	1, 90	1.24	ns
		Interaction	4, 90	0.63	ns
	MI 17-day-old	Colony source	4, 90	1.00	ns
		Transportation	1, 90	6.55	<**0.01**
		Interaction	4, 90	2.91	<**0.03**
Protein content in thorax	GA	Colony source	7, 144	13.10	<**0.01**
		Transportation	1, 144	1.86	ns
		Interaction	7, 144	1.83	ns
Lipid content in abdomen	CA	Colony source	3, 72	2.44	ns
		Transportation	1, 72	0.02	ns
		Interaction	3, 72	4.60	<**0.01**
	GA	Colony source	3, 72	10.54	ns
		Transportation	1, 72	2.47	ns
		Interaction	3, 72	5.09	<**0.01**
	MI 7-day-old	Colony source	5, 108	4.84	<**0.01**
		Transportation	1, 108	2.09	ns
		Interaction	5, 108	1.54	ns
	MI 17-day-old	Colony source	5, 108	1.42	ns
		Transportation	1, 108	2.53	ns
		Interaction	5, 108	1.27	ns

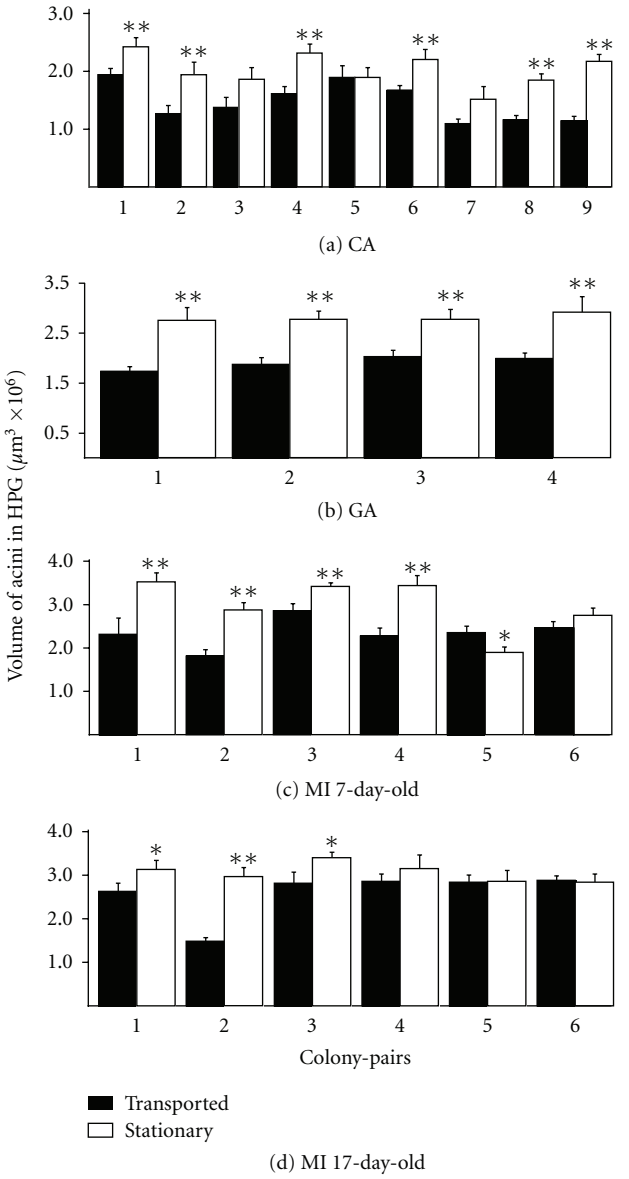

Figure 2: Size of hypopharyngeal glands (mean ± SE) measured as volume of gland acini in worker honey bees experiencing transportation (solid) or no transportation (open) in California (a), Georgia (b), or Michigan ((c), (d)). For more details, see Figure 1 legend.

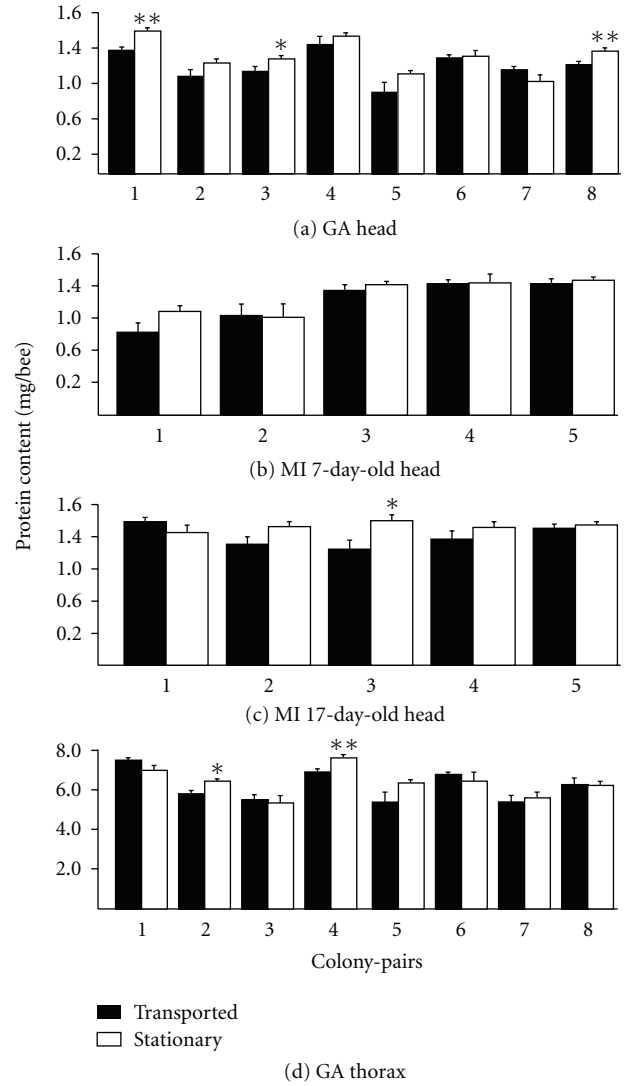

Figure 3: Protein content (mean ± SE) in worker honey bees experiencing transportation (solid) or no transportation (open) in heads ((a), (b), (c)) or thorax (d). For more details, see Figure 1 legend.

no significant differences between the S and T groups in 7-day-old bees.

For 17-day-old bees, there were no significant differences when analyzed together (Table 1), although colony-pair 3 showed significant differences between S and T (Figure 3(c)).

3.4. Protein Content in Thorax

GA Trial. There were no significantly differences between S and T groups in thorax protein content when analyzed together (Table 1), although the S colonies were significantly higher than T colonies in colony-pairs 2 and 4 (Figure 3(d)).

3.5. Lipid Content in Abdomen

CA Trial. There were no significant differences between the S and T groups (Table 1), although colony-pair 3 showed a significant difference between the two groups (Figure 4(a)).

GA Trial. There were no significant differences detected between S and T group when all 4 colony-pairs were analyzed together (Table 1), although S had higher lipid content in abdomen than T in colony-pair 1 (Figure 4(b)).

MI Trial. For 7-day-old bees, there were no significant differences between S and T groups in lipid content in abdomen (Table 1), individual pairs also did not show any differences (Figure 4(c)).

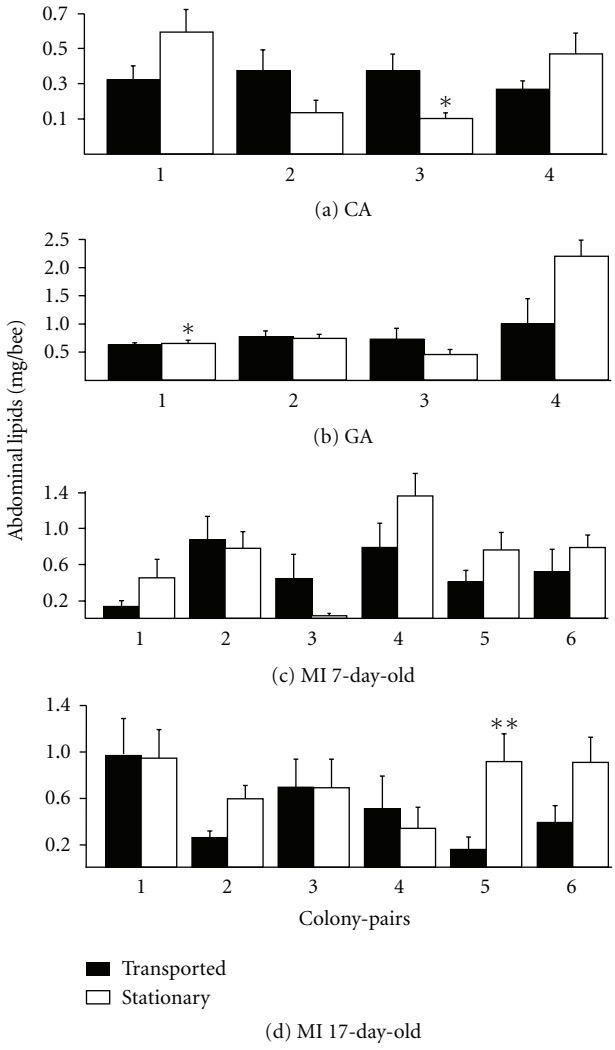

Figure 4: Lipid content (mean ± SE) in worker honey bees experiencing transportation (solid) or no transportation (open) in California (a), Georgia (b), or Michigan ((c), (d)). For more details, see Figure 1 legend.

For 17-day-old bees, there were no significant differences between S and T groups when all 6 colony-pairs were analyzed together (Table 1), although S had higher lipid content in abdomen than T (Figure 4(d)).

4. Discussion

The major finding from this study was that HPG sizes were consistently and negatively affected by transportation. Results showed that the volume of HPG acini were significantly smaller in bees from transported colonies than that from stationary ones (Figure 2). This was true for all three trials conducted in different locations, and also for both young (7-day-old) and old (17-day-old) bees in the Michigan trial. Previous studies have shown that consumption rate of protein diets had a positive correlation with the development of HPG [27, 28]. In this study, the worker bees in T group showed consistently smaller HPG sizes, possibly due to their inability to find or consume pollen normally. It is also possible that trophallaxis was adversely affected such that the initial flow of jelly to very young bees (1–4 days old) from nurses occurred at a lower frequency such that normal HPG development was affected [16]. Yet another possibility is that the queen stopped laying during transportation and the overall level of brood pheromone would be lower in the transported colonies, and this could have affected their HPG development negatively. It is a bit surprising that 17-day-old bees were also affected in their gland size, because these bees were 13 days old when the transportation took place in the MI trial. Workers should have attained their maximum gland size around 12–14 days old [29], however, transportation during this time period still significantly negatively impacted their gland size. It is possible that these 17-day-old bees were actively nursing brood during transportation but they were unable to balance their protein input with proper pollen feeding. This suggests that all in-hive workers (workers that are performing preforaging duties) are affected by transportation. It is puzzling that while HPG acini sizes showed consistent differences in all trials, the head protein or thorax protein content did not show the same pattern. Head protein includes HPG and brain, plus head salivary glands and muscles for the mandibles. Our data here suggest that HPG size responded more consistently to transportation stress. Thorax protein content mainly reflects the mass of the flight muscles, for some reason it also does not show a consistent effect. It is possible that HPGs respond more rapidly, but changes in head or thorax protein content have more variability and do not show the same sensitivity to stress-related events. A recent study also failed to find any differences in bees from healthy and colonies exhibiting colony collapse disorder (CCD) in weights or protein content of head, thorax, abdomen [30].

JH is well studied because it plays many roles in honey bees. It has been known to be involved in the queen-worker caste differentiation during the larval stage [31, 32], in regulating the age-related division of labor in adult workers [33], and in determining aggression levels in workers [34]. JH titers are regulated by the changes in rate of JH biosynthesis and other processes, such as degradation and tissue uptake [35]. A previous study showed that JH levels in foragers also displayed diurnal changes. JH titers were at their lowest just before noon, slightly increased by late afternoon and peaked just before midnight [36]. Lin et al. provided the first evidence that JH titers changed rapidly when workers were removed from their normal social environment and manipulated experimentally [9]. Therefore, JH titers in hemolymph of honey bees were influenced by many factors.

In this study, there were no differences in JH titers between S and T groups, when all colony-pairs were analyzed together by ANOVA. This is contrary to our original hypothesis that transported bees should have higher JH titers than stationary bees. It is possible that our bees were too young ($\leq$8 days in all trials except MI 17-day-old bees) to observe an effect on JH. However, even in 17-day-old bees (Figure 1(d)), only colony-pair 1 showed significantly higher

JH titers in the T group, while colony-pair 2 showed a trend but it was not significantly different. It is possible that JH is affected by too many factors and is not a good indicator for transportation-related stress. Alternatively, the transported bees did not experience a faster behavioral development as we originally hypothesized.

Toth and Robinson found that abdominal lipid stores in honey bees decline prior to the onset of foraging [20]. Before this study, we had hypothesized that lipid content in abdomen should be lower in transported bees, either due to faster behavioral development, or due to less pollen consumption during the long distance transportation. However, the data here showed that there were no significant differences between stationary and transported groups in their fat content. This agrees with the JH data, suggesting that transported bees did not experience a faster behavioral development.

Our study concentrated on younger bees in the colony, assuming that they would be more sensitive to transportation-generated disturbances. However, it might be that the older bees are more sensitive to this process. Foragers, for example, might experience higher mortality due to their higher metabolism, and also due to a lack of jelly fed to them during the transportation, as suggested by this study. We assumed that the physiological responses were maximal immediately after the transportation took place. However, it is possible that it might take 3-4 days for the effect to be manifested and we might therefore have missed effects on accelerated development. We also did not know how long the negative impact lasted on the transported bees or whether their glands would recover after another week. A laboratory proxy for long distance transportation is needed to further dissect the detailed mechanisms of transportation-induced stresses.

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Research Article

Pollination Requirements and the Foraging Behavior of Potential Pollinators of Cultivated Brazil Nut (*Bertholletia excelsa* Bonpl.) Trees in Central Amazon Rainforest

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This study was carried out with cultivated Brazil nut trees (*Bertholletia excelsa* Bonpl., Lecythidaceae) in the Central Amazon rainforest, Brazil, aiming to learn about its pollination requirements, to know the floral visitors of Brazil nut flowers, to investigate their foraging behavior and to determine the main floral visitors of this plant species in commercial plantations. Results showed that *B. excelsa* is predominantly allogamous, but capable of setting fruits by geitonogamy. Nineteen bee species, belonging to two families, visited and collected nectar and/or pollen throughout the day, although the number of bees decreases steeply after 1000 HR. Only 16, out of the 19 bee species observed, succeeded entering the flower and potentially acted as pollinators. However, due to the abundance, flower frequency and foraging behavior of floral visitors, it was concluded that only the species *Eulaema mocsaryi* and *Xylocopa frontalis* could be considered relevant potential pollinators.

1. Introduction

Brazil nut (*Bertholletia excelsa* Bonpl., Lecythidaceae) is native from the Amazon forest occurring in the wild from 5°N to 14°S in Venezuela, Colombia, Peru, Bolivia, Suriname, Guyana, and Brazil [1–3]. It is harvested for its nut, which is extracted from inside the large, rounded and hard-to-break fruit collected on the ground after falling from the trees [4]. Most production is for export comprising an important source of food and income to the indigenous people [5].

Brazil nut is believed to be an allogamous species presenting mellitophilous pollination syndrome, thus depending on biotic pollinators to set fruits [6]. However, little is known about its breeding system and pollination requirements. The blooming period occurs from September to December, peaking in November, and flowers are produced profusely in vertical terminal panicles [6, 7]. The flower is large (c.a. 3.9 cm in length × 3.6 cm in width), zygomorphic, with two to three sepals, and six yellowish petals [6, 8]. It bears a curled hood made of congruent staminodes, called ligule, that in association with the petals form a chamber which conceals stamens, stigma, and nectaries [8, 9]. The large size and strength of the hood restricts and selects flower visitors to medium- and large-sized bees strong enough to uncurl it [7, 8]. Anthers begin to dehisce while the flower is still closed, around 0100 HR-0130 HR and over 90% of anthers are shedding pollen by 0300 HR. Pollen viability ranges from 76% to 86.5% and remained viable until 1400 HR [10, 11]. Anthesis takes place between 0430 HR to 0500 HR, and petals fall off after 24 h. When fecundation does not occur, the pistil drops after 48 h [10]. The ovary bears an average of 20 ovules, and only 0.28 to 0.40% of the flowers produced set

fruits [12, 13]. Fruits take an average of 15 months to mature [7, 14].

There are few studies investigating floral visitors of Brazil nut, and usually they are restricted to the genus level. Prance and Mori [15] stated that the main pollinators of species belonging to the Lecythidaceae family are *Bombus* and *Euglossa* bees. Müller et al. [10], dealing with *B. excelsa*, believe that large-sized bees of the genus *Bombus* are the main pollinators of this species, while a study carried out in Bolivia, suggested that euglossine bees are the effective pollinators [13]. However, a study carried out in the state of Acre, Brazil, points out to bees of the genus *Xylocopa* [16]. Only Nelson et al. [9] in a study nearby the city of Manaus, State of Amazonas, and Maués [7], working close to the city of Belém, State of Pará, have identified the bee species visiting Brazil nut flowers to the species level. In both cases, they were all medium-to large-sized bees: *Eulaema seabrai* (Moure, 1960), *Epicharis rustica* (Olivier, 1789), *Ep. umbraculata* (Fabricius, 1804), *Eulaema nigrita* (Lepeletier, 1841), *El. cingulata* (Fabricius, 1804), in Nelson et al. [9] work, and *Xylocopa frontalis* (Olivier, 1789), *X. aurulenta* (Fabricius, 1804), *Ep. rustica* (Olivier, 1789), *Ep. affinis* (Smith, 1874), *Centris similis* (Fabricius, 1804), *El.nigrita, El. cingulata, Bombus brevivillus* (Franklin, 1913), and *B. transversalis* (Olivier, 1789), in Maués [7] report. Recently, Santos and Absy [17] reported *X. frontalis* and *El. mocsaryi* (Friese, 1899), as the most abundant floral visitors of *B. excelsa* flowers in Itacoatiara county, State of Amazonas.

There is a lack of precise information on the breeding system and floral visitors of *B. excelsa*. This work aimed to investigate the pollination requirements, learn about the identity and foraging behavior of visitors to Brazil nut flowers, and discuss their potential as pollinator of this plant species. Such knowledge is remarkably important in developing policies of sustainable use of the forest and conservation of the native bee pollinators. It may also help to explain and to overcome the low productivity observed in commercial plantations of Brazil nut [8–10].

2. Methods

The experiment was carried out in Aruanã farm, situated on the road Manaus-Itacoatiara, km 215, county of Itacoatiara, State of Amazonas, Brazil, at 3° 0′ 30.63″ S and 58° 50′ 1.50″ W. The farm total area comprises 12,000 ha, of which 3,600 are cultivated with 20 varieties of grafted Brazil nut trees. The trees are spaced at 20 × 20 m reaching approximately 1,300,000 trees. It is the largest Brazil nut plantation in the world.

Four trees (three belonging to variety 609 and one to variety Abufari) were chosen at random out of those in blooming. These trees were ca. 0700 HR apart from each other and ranged from 25–30 m in height. Scaffolds were built by the side of each tree, allowing to spot visually 60% of their canopies and access flowers for data collection. Field observations were carried out for 78 days, from October to December 2007, covering the whole flowering period, especially its peak in November.

2.1. Pollination Requirements. Aiming to know the pollination requirements of Brazil nut trees and the role of bees in pollinating this plant species, we applied five pollination treatments to the trees during their blooming.

T1: Open Pollination. We marked 655 buds with satin threads tied to their petiole in the day before flower anthesis. These buds were observed throughout the anthesis and flower lifespan until they have fallen from the trees or being set, until 25 days later. In this treatment, we aimed to know the natural levels of pollination of Brazil nut trees in the area studied.

T2: Restricted Pollination. 326 buds were covered with muslin bags and remained bagged for 25 days. The aim of this treatment was to verify the dependence or nondependence of Brazil nut flowers on biotic pollination.

T3: Hand Cross-Pollination. 150 buds were marked with satin threads and bagged with muslin bags. Next day, after anthesis, flowers were unbagged and manually pollinated with pollen grains from flowers of another Brazil nut tree being deposited directly on the stigma. Donor flowers were collected minutes before we start to perform hand pollination and taken immediately to receptor tree. Then, pollen grains were removed from the anthers of the donor flower using a fine painting brush and transferred promptly to the stigma of the receptor flower. Immediately after hand-pollinated, the flowers were protected with muslin bags for 25 days. This treatment indicates cross-pollination requirements of the brazil nut tree and the existence any pollination deficit by comparison to natural fruit set in the area (open pollination).

T4: Hand Self-Pollination. We marked 98 buds and followed the same procedure described above, except that pollen grains were transferred between anthers and stigma of the same flowers. In this treatment, results show if the Brazil nut tree is self-compatible or not.

T5: Geitonogamy. The same procedure above was repeated here with 78 buds, but pollen grains were transferred from anthers of a flower to the stigma of a different flower from the same tree. We aimed to learn if the Brazil nut tree shows any sort of incompatibility, this kind of crossing and, its dependence on foreign pollen grains.

In this experiment, colors of the satin threads varied according to the treatment, and satin threads were carefully tied to the buds' petiole avoiding damaging the buds, obstruction of the anthesis, and normal development of the flower and fruit set. Also, all hand pollinations were performed between 0600 HR and 0800 HR when, according to Müller et al. [10], fecundity is greatest.

Brazil nut fruits take an average of 14 months to ripe, and other factors besides pollination can interfere with fruit persistence on trees [7, 14]. Thus, in all tests we assessed initial fruit set 25 days after flower manipulation as a measure of pollination effectiveness. This is a reliable measure because unpollinated flowers fall from the trees in the same day they

open, while pollinated ones remain on the trees and show an ovary about 1.5 mm in diameter 25 days later.

2.2. Floral Visitors and Foraging Behavior. Samples of all floral visitors were collected from each tree using entomological nets at every hour from 0500 HR to 1700 HR. Then, insects were killed in a lethal chamber with ethyl acetate, pinned, identified at species level and, sexed, and counted to determine their specific abundance.

During blooming, the foraging behavior of each flower visiting species was recorded considering the following parameters: frequency, abundance, hour of the day and number of visits, time spent per flower, approach and handling of the flower, and entry to the flower. Data were collected using a notepad, a stop watch, a video and photo camera Sony Cyber-shot DSC-H50 9.1 MP, and by means of visual observation of the bees foraging on the flowers, most of them are out of the reach of the observer but in his sight. Recording was initiated when the bee species arrived to the tree and stopped when the insect flew away or went out of the observer's sight, that was limited to only part of the canopy. All data were collected in 25 periods of 30 minutes each, starting at 0500 HR and ending by 1700 HR. This information was later related to temperature, and air relative humidity records obtained every 30 minutes using a digital thermal hygrometer, model Impac TH02, because there are evidences that increases in ambient temperature have a negative impact o the foraging of bees [18, 19].

2.3. Statistical Approach. Data on pollination requirements did not conform to the ANOVA presumptions due to their binomial character (set fruit or nonset fruit) and were analysed using the nonparametric test of Kruskall-Wallis, and means were compared by the nonparametric Dunn's test.

Data regarding the number of flowers visited per tree and time spent per flower were analysed by ANOVA, and means were compared *a posteriori* by Tukey test at 5%. All tests were performed using SPSS 19 Statistics program.

3. Results

3.1. Pollination Requirements. There were significant ($P < 0.05$) differences between treatments for fruit set (Table 1). The hand cross-pollination treatment set the greater number of fruits and differed ($P < 0.001$; KW = 54.295) from all other treatments, while the geitonogamy treatment did not differ ($P < 0.001$, KW = 54.295) to the free pollination treatment. Flowers submitted to the restricted and hand self-pollination treatments set no fruits (Table 1).

3.2. Flower Visitors and Foraging Behavior. Flowers of *B. excelsa* were visited by a wide range of animals, such as Hymenoptera (bees), Lepidoptera (butterflies and moths), and birds (hummingbirds). In Hymenoptera, a great variety of bee species was observed and collected visiting Brazil nut flowers. These bees belonged to two families (Apidae and Megachilidae) in a total of 19 species (Table 2).

Observations on the foraging behavior of floral visitors and potential pollinators showed that bees collect both pollen and nectar from *B. excelsa* flowers. The place from where bees collected nectar from the flowers varied according to the species size. Larger bee species harvested nectar from the ligule base, while smaller species got inside the flower to collect the nectar present at the base of the anthers.

Table 1: Initial fruit set of Brazil nut (*Bertholletia excelsa*) flowers submitted to five pollination treatments: open pollination, bagged with muslin bags, hand cross-pollination, hand self-pollination, and geitonogamy. Itacoatiara, Amazonas, Brazil, 2007.

Treatments	n	Fruit set (number)	Fruit set (%)
Free pollination	655	20	3.05[b]
Pollinator exclusion	326	0	0
Hand cross-pollination	159	29	19.33[a]
Hand self-pollination	98	0	0
Geitonogamy	78	3	3.85[b]

[*] Values followed by the same letters are not significantly different ($P < 0.001$; Kruskal-Wallis ANOVA).

Bees initiated harvesting pollen and nectar at 0515 HR and reached a peak of foraging activity between 0530 HR and 0600 HR. After 1000 HR the number of bees foraging on flowers dropped steeply, coinciding to the temperature increase and relative air humidity drop (Figure 1). However, a small number of bees kept foraging in the afternoon, specially the species *Xylocopa frontalis*. On the contrary of Müller et al. [10] report of bees starting to forage earlier in the dawns following full moon nights, we did not register any difference from the other nights ($n = 2$).

The most abundant floral visitor of Brazil nut was the carpenter bee *Xylocopa frontalis*. This species was the first one to arrive at the flowers (around 0515 HR) to collect nectar and pollen (Figure 2(a)) and was found in great numbers and frequency throughout the whole blooming season of the trees studied. After reaching a flower, *X. frontalis* was used to make a brief inspection of it and, if not rejected, pushed inside the flower using its ligule as a platform to collect nectar from the base of the ligule itself. This bee species was, apparently, the one which carried more pollen on its body, especially on the back of the thorax, head, and in the scopa. A typical behavior observed in *X. frontalis* while foraging was to sit on a flower and groom pollen out of its body towards the scopa and discard with the forelegs the exceeding pollen grains. *Xylocopa frontalis* was among the three bee species that visited most flowers per tree and spent over than 10 seconds per visit (Table 3). Males were observed visiting flowers for nectar, but they also carried great amounts of pollen on their thorax (Figure 2(b)).

Centris denudans (Lepeletier, 1841) was observed visiting flowers (Figure 2(h)) in the canopy of all trees of this study. It was present throughout the blooming season, carrying small amounts of pollen on the back of the thorax, despite the bee large size. This species frequently chased after other individuals of the same species in quick flights over the canopy, possibly to drive the other bee off the food source or to mate with her. It was one of the few species observed foraging in the afternoon, the hottest part of the day,

Table 2: List of families, species, sex and body size of bees, floral visitors, and potential pollinators of Brazil nut (*Bertholletia excelsa*), collected in a commercial cultivation in the county of Itacoatiara, state of Amazonas, Brazil, 2007.

Family	Species	Sex	Body size (mm) ± s.d.
Apidae	*Xylocopa* (*Neoxylocopa*) *frontalis* (Olivier, 1789)	♂♀	34.60 ± 0.10
Apidae	*Epicharis* (*Epicharana*) *flava* (Friese, 1900)	♀	17.40 ± 0.26
Apidae	*Epicharis* (*Epicharana*) *conica* (Smith, 1874)	♂♀	12.30 ± 0.97
Apidae	*Epicharis* (*Epicharis*) *umbraculata* (Fabricius, 1804)	♀	28.70 ± 1.10
Apidae	*Epicharis* (*Parepicharis*) *zonata* (Smith, 1854)	♀	15.20 ± 0.75
Apidae	*Centris* (*Ptilotopus*) *americana* (Klug, 1810)	♀	35.10 ± 0.88
Apidae	*Centris* (*Trachina*) *carrikeri* (Cockerell, 1919)	♂	5.50 ± 1.04
Apidae	*Centris* (*Xanthemisia*) *ferruginea* (Lepeletier, 1841)	♀	7.80 ± 0.45
Apidae	*Centris* (*Ptilotopus*) *denudans* (Lepeletier, 1841)	♂♀	34.20 ± 1.75
Apidae	*Eulaema* (*Eulaema*) *meriana* (Olivier, 1789)	♂♀	33.40 ± 1.20
Apidae	*Eulaema* (*Apeulaema*) *mocsaryi* (Friese, 1899)	♂♀	15.60 ± 0.84
Apidae	*Eulaema* (*Apeulaema*) *cingulata* (Fabricius, 1804)	♀	14.60 ± 0.93
Apidae	*Bombus* (*Fervidobombus*) *transversalis* (Olivier, 1789)	♀	16.40 ± 2.86
Apidae	*Eufrisea purpurata* (Mocsáry, 1896)	♀	10.80 ± 0.89
Apidae	*Eufrisea flaviventris* (Friese, 1899)	♀	15.30 ± 1.33
Apidae	*Apis mellifera scutellata* (Lepeletier, 1836)	♀	4.40 ± 0.19
Apidae	*Frieseomelitta longipes* (Smith, 1854)	♀	1.50 ± 0.24
Apidae	*Melipona* (*Michmelia*) *lateralis* (Erichson, 1848)	♀	4.90 ± 0.32
Megachilidae	*Megachile* sp. 1	♀	4.65 ± 0.76

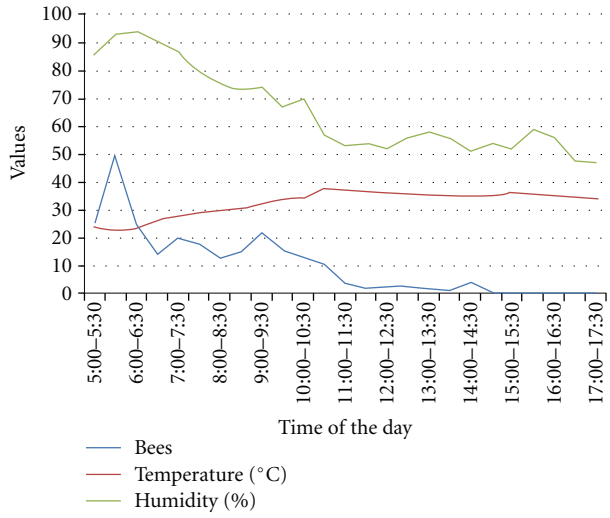

Figure 1: Frequency of floral visitors associated to temperature and relative humidity (at each 30 minutes) in a commercial cultivation of Brazil nut (*Bertholletia excelsa*) in the county of Itacoatiara, state of Amazonas, Brazil, 2007.

although most of its foraging activities were recorded in the morning. This bee species approached the flowers in a different way of *X. frontalis* because it did not inspect and rarely rejected a flower, entering the flower immediately after reaching it, but also harvested nectar from the ligule base. *Centris denudans* ranked second among the species that visited most flowers per tree, usually flowers close to each other, and also spent over than 10 seconds per flower visit (Table 3). Males were observed and recorded visiting *B. excelsa* flowers, and mating events on Brazil nut flowers were also registered.

Eulaema meriana (Olivier, 1789) was also present throughout the blooming season, but only in the morning. Like *X. frontalis*, frequently rejected some flowers but always carried large amounts of pollen in its corbicula. Due to its large glossa, this bee species also collected nectar from the ligule base landing on the ligule itself (Figure 2(k)). *El. meriana* was the bee species that visited most flowers per tree, usually neighboring flowers, spending over than 16 seconds per visit (Table 3). Males of this species were observed harvesting nectar from the Brazil nut flowers.

Centris americana (Klug, 1810) was seen only in some moments of the blooming period and always in small numbers and low frequency to flowers, never exceeding one individual per tree at a given time. This species approached the flower like the other large-sized bees, using the ligule as a platform for landing and collecting nectar from the ligule base (Figure 2(j)). It spent less than 8 seconds per visit (Table 3).

Bombus transversalis was recorded only in the beginning of the blooming season (Figure 2(o)). It was one of the species that spent most time per flower visit, reaching up to 90 seconds inside a flower in some visits. Despite staying long in the flower, *B. transversalis* usually transported small

Figure 2: Approach to flowers of Brazil nut (*Bertholletia excelsa*) by distinct bee species in a commercial cultivation in the county of Itacoatiara, state of Amazonas, Brazil, 2007. ((a); (b)) *Xylocopa frontalis* (♀ and ♂, resp.); (c) *Epicharis (Epicharana) flava* (♀); ((d); (e)) *Epicharis (Epicharana) conica* (♀ and ♂, resp.); (f) *Epicharis (Epicharis) umbraculata* (♀); (g) *Epicharis (Parepicharis) zonata* (♀); (h) *Centris (Ptilotopus) denudans* (♀); (i) *Centris ferruginea* (♀); (j) *Centris (Ptilotopus) americana* (♀); (k) *Eulaema (Eulaema) meriana* (♀); ((l); (m)) *Eulaema* (*Apeulaema*) *mocsaryi* (♂ and ♀, resp.); (n) *Eulaema* (*Apeulaema*) *cingulata* (♀); (o) *Bombus* (*Fervidobombus*) *transversalis* (♀); (p) *Eufriesea flaviventris* (♀); (q) *Megachile* sp.1; (r) *Frieseomelitta longipes* robbing pollen from *El.* (A.) *mocsaryi*.

amounts of pollen and visited only a few flowers per tree (Table 3). Due to its medium size, this species entered almost entirely in the flower to collect nectar at the ligule base.

Eulaema mocsaryi was the second most abundant and frequent species over the whole blooming season, mainly in the morning shift (Figure 2(m)) but also observed visiting flowers in the afternoon. It frequently rejected flowers that possibly had been previously visited by other bee. Between two flower visits, while in flight or landing on a leaf, individuals of this species combed pollen from their bodies into the corbicula making large pollen loads. This bee visited less than five flowers per tree moving quickly to other trees

Table 3: Bee relative abundance, mean number (± standard error: SE) of flowers visited per tree by ten bee species and mean time (± standard error: SE), in seconds, spent by twelve bee species per visit to flowers of Brazil nut (*Bertholletia excelsa*) variety 609, under cultivation in the Amazon rainforest (*n*: number of bees recorded per species).

Species	Relative abundance	Number of flower visits per tree			Time spent per flower visit		
	(%)	*n*	X ± S.E.		*n*	X ± S.E.	
Xylocopa frontalis	62.85	136	11.33 ± 0.834	abc	64	11.63 ± 0.754	bcd
Centris denudans	6.84	35	14.71 ± 2.368	bc	64	11.96 ± 0.736	bcd
Centris americana	1.11		—		4	7.73 ± 0.694	cd
Centris ferruginea	0.55	3	3.67 ± 2.667	c	31	9.14 ± 0.854	cd
Eulaema meriana	6.65	17	15.10 ± 2.358	a	57	16.05 ± 1.204	bc
Eulaema mocsaryi	12.20	72	4.36 ± 0.514	abc	48	15.34 ± 1.488	bc
Eulaema mocsaryi (male)		9	8.33 ± 2.677	abc	55	5.68 ± 0.265	d
Epicharis conica	3.88	8	2.75 ± 0.773	c	7	18.39 ± 2.714	bcd
Epicharis flava	0.37	7	4.43 ± 1.288	bc	45	11.86 ± 1.354	bcd
Epicharis zonata	0.92	9	1.67 ± 0.289	c	3	31.38 ± 13.090	a
Eufrisea flaviventris	0.37	6	7.33 ± 3.373	abc	58	5.96 ± 0.983	d
Eufrisea purpurata	0.74		—		4	14.54 ± 5.809	bcd
Bombus tranversalis	3.51	3	6.33 ± 2.963	abc	42	27.61 ± 1.928	a

* Values followed by the same letters are not significantly different ($P < 0.005$; ANOVA).

(Table 3). However, when visiting a flower, *El. mocsaryi* spent over 15 seconds increasing the chance to deposit pollen on the stigma (Table 3).

Epicharis conica (Smith, 1874) was present throughout the blooming season and like *El. mocsaryi* was more frequent in the morning shift, but also present in the afternoon. Due to its small size, this species penetrates the flower almost entirely and unlike the previous species present here, the bee makes a turn inside the flower before leaving it facing out (Figure 2(d)). This bee was the second species that visited less flowers per plant, but took over 18 seconds per visit ($n = 7$) (Table 3). Males also visited flowers and pushed their bodies completely through the petals getting hidden by the ligule while inside the flower (Figure 2(e)). Because of this behavior, their presence was only noticed because the buzzing noisy produced when approaching the flower.

Epicharis flava (Friese, 1900) was present in reduced numbers and only when most trees were in bloom. It carried much pollen on the back of the thorax (Figure 2(c)), outstanding as a potential pollinator of Brazil nut flowers. This bee visited few flowers per tree and spent around 12 seconds per visit (Table 3).

Epicharis zonata (Smith, 1854) is a small bee that like other species of its size gets inside the flower becoming hidden from sight and leaves it facing out carrying small amounts of pollen on its body (Figure 2(g)). This bee was only found in the peak of the blooming season, mainly around 0900 HR. It is a fast-flying bee that moves between trees frequently making difficult to track its path over a single tree canopy. As a consequence, *Ep. zonata* produced the smaller number of flowers visited per tree among all bee species observed in this study, compensated for the longest period of time registered for flower visit (Table 3).

Eufriesea flaviventris (Friese, 1899) is a medium-sized, fast-flying species, and the faster flower visitor observed in this study spending around only six seconds per visit (Table 3), but many times revisiting consecutively the same Brazil nut flower. This was the only species observed to collect exclusively pollen (Figure 2(p)). It also rejected flowers previously visited by other bees and combed the pollen from its thorax to the corbicula while in flight.

Centris ferruginea (Lepeletier, 1841) is a fast-flying, small-sized bee that penetrates the flower almost entirely using the ligule as a platform. It also leaves the flower facing out (Figure 2(i)) and carrying small amounts of pollen on the back of the thorax. Usually was only noticed due to the buzzing sound of its flight over the canopy. This bee species also visited few flowers per tree, favoring cross-pollination (Table 3).

Megachile sp. was the smaller species registered visiting Brazil nut flowers in this study. It penetrated entirely the flower pushing its body among the petals and ligule and also left the flower facing out with small amounts of pollen on its ventral scopa (Figure 2(q)). Because of its size, probably collected nectar from the base of the anthers and stigma, although it s not possible to know for sure because the bee remained hidden inside the flower while sipping nectar. Due to its low frequency and high flight speed, only one visit was registered.

Eulaema cingulata, *Epicharis umbraculata*, *Centris carrikeri* (Cockerell, 1919), and *Eufriesea purpurata* (Mocsáry, 1896) were collected and observed visiting Brazil nut flowers; however, only in rare occasions not allowing even photos to be taken for the two latter species.

Melipona lateralis (Erichson, 1848) was seen only once visiting a flower and captured immediately after leaving the

flower. No further sights were possible until the end of the study.

Apis mellifera scutellata (Lepeletier, 1836) was the only nonnative species recorded in this study, constituting an invading bee in the Amazon ecosystem. It was present in small numbers flying over the canopy, mainly early in the morning. Because of its small size and strength, the bee could not pull the ligule back as a platform as did the larger bee species or push herself among the ligule and petals to get inside the flower as done by other medium and small-sized bees and remained flying over the flowers and landing to collect small amounts of pollen fallen on petals or ligule after the visits by larger bees.

Frieseomelitta longipes (Smith, 1854) was found in the trees all over the morning shift and in greater numbers than *A. mellifera* and, for the same reasons, also did not get assessment of the floral resources inside the flower. However, *F. longipes* showed the behavior of trying to rob pollen from the corbicula of large bees in the moment they were visiting the flowers (Figure 2(r)), sometimes making these bees to give up the flower.

Besides bees, butterflies, hawk moths, and hummingbirds were also seen visiting Brazil nut flowers. Butterflies use to land on the flower and insert their long proboscis to collect nectar at any time of the day. Hawk moths were only present early in the morning, around 0430 HR. They hovered in front of the flowers and introduced their proboscis through the petals to collect nectar. Hummingbirds showed no preference for time of the day, visiting flowers at any time and also hovered in front of the flowers to introduce their beak and drink nectar.

4. Discussion

Results showed that *B. excelsa* did not set any fruit in the restricted and hand self-pollination treatments suggesting that this species cannot bear fruits from pollen grains originated from the same flower and requires biotic pollinators to transfer pollen grains between flowers. According to Moritz and Müller et al. [6, 10] the Brazil nut tree does not set from self-fertilization because this mating system led to less than the 85% ovule fertilization necessary for fruit set. However, the geitonogamy treatment produced over 3% of fruit set indicating that the Brazil nut tree can set fruits when pollen grains are transferred between flowers of the same plant. Also, results of the geitonogamy treatment were similar to the open pollination treatment signifying that the pollination achieved in this commercial plantation could be accounted to geitonogamy. These findings, associated to the much greater fruit set following hand cross pollination indicates that the Brazil nut tree is an allogamous species, in accordance to other authors [6, 8–10].

Our results may explain why the individual plant production is much higher in natural clusters of few Brazil nut trees in the forest than in plantations with hundred of trees. In the natural environment, with much fewer flowers to visit, pollinators may be forced to move between trees and revisit flowers in a much more frequent fashion than when they face a seemly unlimited number of blooming trees.

Although many species visit Brazil nut flowers, only some bee species showed foraging behavior compatible to potential pollinators of this tree. While bees were numerous and concentrated their visits to the morning shift, when flowers presented fresh pollen and were more receptive [10], butterflies, and hummingbirds visited inflorescences at any time of the day, in an inconstant pattern and in low numbers. Hawk moths, however, visited flowers in the dawn, close to the sunrise, but were also scarce. Besides that, the great majority of bee species entered and moved inside the flower increasing the chance to transfer pollen from their bodies to the stigmas, while butterflies, hawk moths, and hummingbirds remained outside the flower and introduced a much smaller portion of their bodies, proboscis for the Lepidoptera and beak to the bird, being less likely to deliver pollen to the stigmas. This behavior, in association to the reduced number of individuals, erratic foraging activities, and time of flower visit, suggests that these groups of floral visitors play little or no role in the pollination of *B. excelsa*. On the contrary, the foraging behavior of most bee species indicates that they can be effective pollinators of Brazil nut flowers, in accordance with the suggestions of Prance and Mori [15], Maués and Oliveira [20], Maués [7], Zuidema [13], and Argolo and Wadt [16].

However, some bee species could not enter the flower or did not show a behavior suggestive of relevant pollinators for Brazil nut. The behavior of *Epicharis conica, Ep. zonata, Megachile* sp., and *Centris ferruginea* approaching the flower facing in and leaving it facing out after turning its body inside the flower can contribute to considerable deposition on the stigma of the flower's self-pollen (self-pollination), showed here to produce no fruits. It may happen because the bee leaving the flower facing out can touch the stigma with the back of its thorax, where the pollen has just been placed by the anthers, resulting, at the best, in a mixture of the pollen bees carried from previously visited flowers with that presently visited being deposited on the stigma. In such a situation, these bee species would not be efficient pollinators of Brazil nut flowers because *B. excelsa* is a predominantly allogamous species [6, 7]. Also, *Apis mellifera, Melipona lateralis*, and *Frieseomelitta longipes* did not manage to enter the flowers and could not pollinate them. Besides that, *F. longipes* sometimes prevented flowers to be visited by legitimate pollinators chasing them away for attempting to rob pollen from their corbicula. Although this specific behavior had not been reported before, Santos and Absy [17] showed that the presence of other insects on the flowers can make some floral visitors, presumably pollinators, to avoid these flowers.

Despite potential pollinators, the rare visits of *Eulaema cingulata*, *Epicharis umbraculata*, *Centris carrikeri*, and *Eufriesea purpurata* to Brazil nut flowers suggest that these species contribute little to the pollination of *B. excelsa*. But their presence in the trees may explain why Zuidema [13] pointed out euglossine bees as likely pollinators of *B. excelsa*, although *E. umbraculata*, *C. carrikeri*, and *E. purpurata* had never before been reported as floral visitors of Brazil nut flowers and *E. cingulata* only once in the study by Maués [7].

Although bees of the genus *Bombus* had been suggested as the main pollinators of *B. excelsa* [10, 12], in the present

study only one *Bombus* species, *B. transversalis*, visited the Brazil nut flowers. Nevertheless, these visits were limited to the onset of the blooming season. Therefore, it is likely that the genus *Bombus* does not consist in a relevant taxon for the pollination of *B. excelsa* in the area studied here. Similarly, *Epicharis flava* and *Centris americana* were not abundant and were selective in relation to the blooming stage and probably are not among the main pollinators of Brazil nut flowers.

Bee species like *Centris denudans*, *Eulaema meriana*, *Eufriesea flaviventris*, *Xylocopa frontalis*, and *Eulaema mocsaryi* were frequent in the area during most of the blooming season and showed body size and flower handling adequate to pollinate *B. excelsa* flowers. However, due to the abundance and foraging behavior in the trees, we identified *Eulaema mocsaryi* and *Xylocopa frontalis* as the most relevant pollinators of cultivated *B. excelsa* in Central Amazonia. It is important to stress that, although these two bee species are the ones that potentially most contribute to Brazil nut pollination under the conditions found in this study, the pollination level achieved in the plantation is the sum of the pollination performed by each bee species that constitute that guild of pollinators, including those species that contributed less to the process [21, 22].

Many of the bee species presented in this study as floral visitors and potential pollinators of Brazil nut are widespread in the Amazon region, and some of them also occur in other Brazilian ecosystems [23–25]. Some of these bee species were also reported in the literature interacting with other plant species and constitute important floral visitors or even pollinators. *Eulaema cingulata* is a pollinator to *Ischnosiphon gracilis* (Rudge) Koern (Marantaceae) and floral visitor of *Solanum stramonifolium* Jacq. (Solanaceae) in a fragment of the Atlantic Forest in NE, Brazil [23, 24]. Vilhena and Augusto [25] identified *Ep. flava* as an important floral visitor of *Malpighia emarginata* in a cerrado area of Central Brazil.

In the Amazon, studies carried out in the same area of this work on the floral biology of *Bellucia grossularioides* (Melastomataceae) and floral visitors of *Bixa orellana* (Bixaceae) reported *El. mocsaryi* and *X. frontalis* as the main visitors of these plant species [26, 27]. However, only recently Santos [17] produced the first report suggesting *E. mocsaryi* as an important floral visitor and potential pollinator of *B. excelsa*. Males of *Eulaema meriana* were observed in the present work visiting flowers of Brazil nut to feed on nectar. According to Williams and Whitten [28], these male bees are pollinators of *Catasetum tricomis* (Orchidaceae), suggesting some level of interdependence among these three species because the orchid provides only essences for the male bees of *Eulaema meriana* attract their conspecific females, but the pollen and nectar necessary for the bee survival and reproduction got to be obtained from other plant species, like the Brazil nut.

These observations support the claim of Kremen et al. [29, 30] that conserving the native vegetation on the surrounding of cultivated areas is essential to keep stable populations of pollinators, such as the bees of the present study, for providing food, nesting, and other resources indispensable for their survival. The lack of effective pollinators in numbers adequate to pollinate the large number of flowers present in commercial plantations of Brazil nut can be a cause for the low tree productivity observed in these areas. Among all species identified as potential pollinators of *B. excelsa* in this study only *X. frontalis* have been reared in rational nest boxes and tentatively managed for pollination of passion fruit (*Passiflora edulis* Sims. f. *flavicarpa* Deg.) in NE Brazil [31]. Investigations on the possibility of rearing and managing *X. frontalis* and other species identified here for pollination of *B. excelsa* are needed.

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