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REVIEW ARTICLE

Standard methods for pollination research with *Apis mellifera*

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Summary

In this chapter we present a synthesis of recommendations for conducting field experiments with honey bees in the context of agricultural pollination. We begin with an overview of methods for determining the mating system requirements of plants and the efficacy of specific pollinators. We describe methods for evaluating the pollen-vectoring capacity of bees at the level of individuals or colonies and follow with methods for determining optimum colony field stocking densities. We include sections for determining post-harvest effects of pollination, the effects of colony management (including glasshouse enclosure) on bee pollination performance, and a brief section on considerations about pesticides and their impact on pollinator performance. A final section gives guidance on determining the economic valuation of honey bee colony inputs at the scale of the farm or region.

Métodos estándar para el estudio de polinización con *Apis mellifera*

Resumen

En este capítulo se presenta una síntesis de las recomendaciones para la realización de experimentos de campo con abejas melíferas en el contexto de la polinización agrícola. Comienza con una revisión de los métodos para la determinación de los requisitos del sistema de reproducción de las plantas y de la eficacia de los polinizadores específicos. Se describen métodos para evaluar la capacidad de las abejas como vectores de polen a los niveles de individuos o de colonias, y se continúa con los métodos para la determinación de las densidades óptimas de colonias en campo. Se incluyen secciones para la determinación de los efectos de la polinización en la cosecha, los efectos del manejo de las colonias (incluyendo el cercado en invernaderos) en el rendimiento de polinización de las abejas, y una breve sección sobre consideraciones acerca de los plaguicidas y su impacto en el rendimiento de los polinizadores. Una última sección ofrece una guía para la determinación del valor económico de los gastos de las colonias de abejas melíferas a escala de explotación o de región.

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西方蜜蜂授粉研究的标准方法

摘要

本章给出了蜜蜂授粉田间试验的综合推荐规范。文章开篇概述了测定植物交配系统需求和测定特定授粉者效率的方法。介绍了在个体或群体水平评估蜜蜂的花粉媒介能力的方法，以及确定蜂群田间最适饲养密度的方法。本章节还包括测定授粉的“采摘后”效应和蜂群管理（包括温室环境）对蜜蜂授粉表现的影响，并且简要叙述了对于农药的担忧及农药对授粉表现的影响。最后一节给出了如何在农场或地区层面测定蜂群贡献的经济价值。

Keywords: COLOSS, *BEEBOOK*, honey bee, *Apis mellifera*, pollination, pollen load, pollen deposition, pollination efficacy, pollinator density, crop pollination metrics, pesticides, greenhouse, economic value

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1. Introduction

This chapter describes field and lab procedures for doing experiments on honey bee pollination. Most of the methods apply to any insect for which pollen vectoring capacity is the question. What makes honey bee pollination distinctive is its historic emphasis on agricultural applications; hence one finds a preoccupation with matters of bee densities, behaviours, and management with a view to optimizing crop yields and quality. However, the same methods can be modified to address broader questions on plant fitness and ecosystem-level interactions.

2. Plant pollination requirements

The impact of any pollinator, whether in terms of agricultural production or plant fitness, is an interaction between at least two dynamics: the pollen vectoring capacity of the flower visitor and the genetic obligation, or responsiveness, of the plant to pollen deposition on its stigmas (Delaplane, 2011). Most of this chapter is devoted to appraising pollen vectoring capacity, but in this section we begin with the underlying demands of the plant because this is the necessary starting point for understanding and contextualizing any pollination syndrome: the suite of flower characters derived by natural selection in response to pollinating agents, whether biotic or abiotic (see Faegri and Pijl, 1979).

To begin, *pollination* is the transfer of pollen from the anthers to the stigma of flowers of the same species and is essential to the reproduction of most angiosperms (flowering plants). Pollination success is often measured in terms of percentage fruit- or seed- set. Fruit- or seed-set is the ratio of ripe fruit or seeds relative to initial number of available flowers or ovules, respectively. This ratio is rarely 100% owing to such factors as normal levels of fruit abortion, suboptimal pollination conditions, herbivory, or cultural problems.

The degree to which a plant species depends on a particular pollinator is determined in part by the mating and breeding system of the plant (Fig. 1). Some plants can produce seeds or fruits without pollination, and understanding this process is important for understanding when the honey bee can or cannot contribute to fruit- or seed-set and yield enhancement. Asexual reproduction through non-fertilized seeds is called *apomixis* or *agamospermy*. Apomixis happens when an embryo is formed either from an unfertilized egg within a diploid embryo sac that was formed without completing meiosis (blackberries, dandelions) or from the diploid nucleus tissue surrounding the embryo sac (some *Citrus* species, some mango varieties). When fruit forms without fertilization of ovules, either naturally or chemically-induced, this is called *vegetative parthenocarpy* (banana, pineapple, seedless cucumber). In either apomixis or parthenocarpy no fertilization occurs, and pollination is not required. However, in some plant species, pollination or some other stimulation is required to produce parthenocarpic fruits, a chief example being seedless watermelon, a type of stimulative parthenocarpy. Also, in many apomitic plants apomixis does not always occur, or occurs only partially, and sexual reproduction can also take place (*Citrus* and mango).

Most angiosperms, however, need pollination to set seeds and fruits, and with the exception of those whose flowers are capable of autopollinating (ex. many beans, soybean, peach, peanuts), they rely on agents to vector the pollen. Angiosperms have basically two mating systems: outcrossing (*xenogamy*) in which pollination occurs between plants with different genetic constitutions, or selfing (*autogamy*) in which no mixing of different genetic material occurs other than through recombination. Outcrossing is achieved by *cross pollination*, resulting from the transfer of pollen between different flowers of different plants of the same species, while *selfing* is the outcome of pollen transfer within the same flower (*self-pollination*) or between different flowers of the same plant (*geitonogamy*). Some plant species are strictly xenogamous while others are autogamous, but mixed mating systems in which plants use outcrossing and autogamy or even outcrossing, autogamy and agamospermy are not uncommon (Rizzardo *et al.*, 2012).

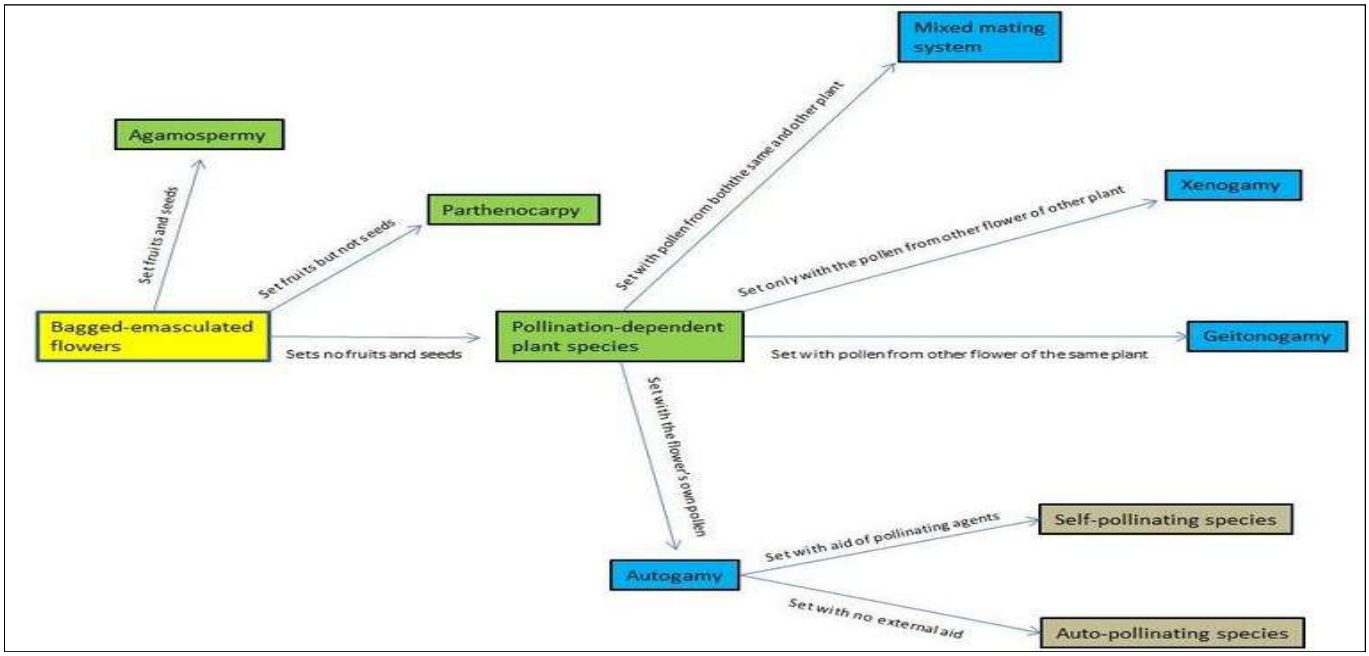

Fig. 1. Plant mating systems and pollination requirements.

The extent to which an angiosperm responds to pollination and the fraction of that pollination that is selfed or out-crossed vary greatly by plant species or variety, and in any particular case a flower visitor must meet specific needs to qualify as a legitimate pollinator. We describe below some field methods for determining the mating system and pollination requirements of plants and the potential pollination role of abiotic and biotic agents, focusing on the level of the individual plant rather than the plant population and drawing heavily upon the following published works (Spears, 1983; Mesquida *et al.*, 1988; Freitas and Paxton, 1996, 1998; Sampson and Cane, 2000; Dafni *et al.,* 2005; Pierre *et al.*, 2010, Vaissière *et al.*, 2011).

2.1. Determining plant mating system

When trying to determine a plant mating system, one can use each of the methods described here as experimental treatments or select only those that appear most relevant to the plant species of interest. In all cases, a positive control in which flowers are marked but otherwise left available for open pollination is necessary to provide a reference for comparison with the manipulative treatments (Fig. 2). In some cases it is also necessary to provide a negative control in which flowers are excluded from all flower visitors for the duration of their dehiscence. It is preferable to reduce background variation by applying distinct treatments to flowers of the same inflorescence, branch, or plant depending on flower abundance and size of the plant.

In the following sections, the performance of a pollinator is implied by the field-scale observation of subsequent fruit- or seed-set. It is also appropriate to measure pollen vectoring capacity at the level of viable pollen on the bee and pollen deposited by the bee onto the stigma. These techniques are covered in sections 3.1. and 3.2.

Fig. 2. Open pollination treatment in soybean plantation: flowers are marked and left open for floral visitors.

2.1.1. Testing for agamospermy (asexual reproduction through non-fertilized seeds)

This test will tell us whether a plant species sets seeds without pollination. If this is so, honey bees cannot contribute to seed- or fruit-set.

1. Choose a given number of flower buds prior to anthesis. The number of buds may vary with availability and ease of access, but larger samples produce more reliable results.
2. Protect half of these buds with pollination bags (Fig. 3) and leave the other half unbagged as a control. Pollination bags are typically made of sheer nylon or similar fine fabric that excludes insects but permits entry of air and light. They are usually semi-transparent nylon and have draw strings to secure the bag around the flower pedicel. The flower should be positioned as much as possible in the centre of the bag so that the mesh does not touch the flower, which could lead to self-pollination. To limit self-pollination further, fix a wire frame around the flower and place the bag over the frame, thus providing structural support to the bag. Identify each treatment with weather-resistant tags. Testing for agamospermy can also be done in a greenhouse without exclusion bags and is thereby easier.

Fig. 3. Restricted pollination treatment: a watermelon flower is bagged throughout its life to prevent honey bee visitation.

3. Before anther dehiscence (depending on the flower species this may happen prior to anthesis), remove the bag (Fig. 4) and emasculate the flower using a fine pair of forceps to minimize injury to floral tissue. After emasculation, replace the pollination bag on the flower to prevent undesired action of pollinating agents. The bags should remain on the flowers while the stigmas are receptive and can be removed afterwards. It is important for the investigator to become familiar with the time of day or floral morphology stage that are conducive to stigma receptivity for a given plant species.
4. After ovule maturation is apparent in the pollinated treatments, check whether fruit has developed from the bagged and emasculated flowers. If none is present, one can conclude that the plant species does not exhibit agamospermy. If fruit does develop, it is necessary to wait until fruit ripening to check for seeds because some plants are parthenocarpic (produce fruits with no seeds and do not depend on pollination). If seeds are set, compare the number of fruits and seeds set per fruit from the emasculated and bagged flowers with those from the control treatment to estimate the proportion of seeds set by agamospermy in that particular plant species.
5. It is important that assessments of seed- or fruit-set occur as early as possible to minimize underestimating set because of losses that occur between set and harvest.

Fig. 4. Unbagging watermelon flower for hand pollination.

2.1.2. Testing for autogamy (auto- or self-pollination)

This test will tell us whether the flower can set seeds and fruits from its own pollen. In such a situation, the contribution of flower visitors may be little or none, but even in auto-pollinating plants the movements of bees inside the flower can sometimes optimize pollen transfer from anthers to the stigma and increase fruit- or seed-set. Auto-pollination or self-pollination should be distinguished from geitonogamy (see section 2.1.3.). Auto-pollination is associated with hermaphroditic flowers and pollen transfer within that flower that is automatic (soybean) or pollinator-optimized, whereas geitonogamy could apply to monoecious plants in which pollen is self-compatible but the actions of a pollen vector are nevertheless needed.

1. Choose a given number of flower buds prior to anthesis. The investigator must become familiar with the flowering pattern of the model plant because flowers in some species open and close more than once, making anthesis difficult to determine. The number of buds may vary with availability and ease of access, but larger samples produce more reliable results.
2. Protect two thirds of these buds with pollination bags (see section 2.1.1.) and leave the other third unbagged as open controls, or in the case of pollinator shortage pollinate these flowers manually with pollen from another plant of the same species. Identify each treatment with weather-resistant tags.
3. After anther dehiscence and when stigmas are receptive, remove the bags of half of the protected flowers (one third of the total marked buds) and hand-pollinate the stigmas with a soft brush using pollen from the anthers of the same flower. Dehiscence can usually be recognized as anthers with a split in the anther wall, pore, or flap that is exposing the pollen. After hand-pollinating, re-bag the flowers to prevent flower visitors or wind pollination. Leave bags on flowers until they are no longer receptive, then remove the bags.
4. At the end of the season, check whether fruit developed from the flowers that remained bagged throughout the experiment. If all or most of these flowers have developed into fruit, the plant species is autogamous and its flowers are capable of auto-pollinating. Honey bees can contribute little to increasing fruit- or seed-set. If only the hand-pollinated flowers developed into fruits, this means that the plant species is autogamous but flowers need a pollinating agent to transfer the pollen grains from their anthers to the stigmas within the flower. In this case, honey bees may be of great value. The proportion

of fruit- or seed-set obtained from the bagged treatment in comparison to the hand-pollinated treatment will tell the comparative strength of autogamy in this plant species (strictly autogamous, highly autogamous, etc.). If no bagged flowers produce fruit or seeds, this means the species may be self-incompatible and probably needs cross pollen to set fruits and seeds. However, sometimes a few fruits or seeds can set even in self-incompatible plants because self-recognition can be incomplete. But in this case, there is little variation in fruit- or seed-set among the treated plants. One should not confound self-incompatibility with self-sterility resulting from inbreeding depression because in the latter case seed set varies greatly among treated flowers, ranging from low values in more inbred plants to high values in less inbred ones. Confirmation of self-incompatibility must be done by examining pollen tube growth in the pistil, a subject covered in section 3.1.5.

2.1.3. Testing for geitonogamy (selfing within the same plant)

Some flower species do not set fruits/seeds when self-pollinated but do so when receiving pollen from other flowers of the same plant (geitonogamy). This can happen between perfect (hermaphroditic) flowers, but it is obligatory in autogamous plants which are monoecious (unisexual male and female flowers on the same plant). This test will tell us whether the flower can set seeds and fruits when receiving pollen from other flowers of the same plant. This is important information because honey bees tend to explore many flowers per plant before moving to other plants, and this behaviour favours geitonogamy.

1. Repeat the procedures for testing for autogamy (section 2.1.2.), but replace the treatment using the flower's own pollen for a treatment using pollen from another flower on the same plant.
2. Conclusions are similar to those above for testing autogamy (section 2.1.2.), except that if fruits or seeds developed from the geitonogamy treatment it means that the plant sets when pollen is transferred between its own flowers. The proportion of fruit- or seed-set obtained from the geitonogamy treatment in relation to the control treatment will tell the extent to which the plant is responsive to this mode of mating system.

2.1.4. Testing for xenogamy (reliance on out-crossing)

In xenogamy, or cross-pollination, the transfer of pollen to the stigma must occur between plants with different genetic constitutions; the result is offspring with greater genetic diversity than those for species exhibiting self-pollination or geitonogamy. Cross-pollination is also important because some plant varieties, genotypes, and even individuals are entirely self-incompatible and obligated to receive pollen from another variety, genotype, or individual to set fruits. Even self-fertile plants may produce more fruit or seeds of better quality when cross-pollinated than when self-pollinated, and the extent of this can be determined if outcrossing and selfing (within flower / within plant) are tested at the same time. Crops which grow from highly outcrossed seeds are often more vigorous than ones grown from inbred seeds. Finally, xenogamy is of paramount importance for the production of hybrid varieties and hybrid seed, both of which are of increasing importance.

Knowing the extent to which a plant is obligated to xenogamy helps researchers and growers manage bees optimally and combine compatible cross-pollinating varieties (called *pollinisers*) to promote high rates of pollen transfer (see Jay, 1986; Free, 1993).

1. Repeat the procedures for testing for autogamy (section 2.1.2.), but replace the treatment using the flower's own pollen for one using pollen from a flower of a different plant. In order to prevent using genetically related pollen (parents or siblings), do not collect pollen from plants close to the one whose flowers will be tested.
2. In order to identify compatible pollinisers, the experimental design requires a systematic selection and application of pollen from a number of different varieties of the same plant species. Finding compatible pollinisers is crucial for many commercially important crops such as almond, apple, and plum and is a standard feature of commercial grower guides for planning orchard plantations.
3. Conclusions are similar to those when testing for autogamy (section 2.1.2.), except that if only the xenogamy treatment develops fruit, the plant species is xenogamous and its flowers need a pollinating agent to transfer pollen between flowers of different plants. In this case, honey bees can be of great value. The proportion of fruit- or seed-set obtained from the cross pollination treatment in comparison to the control will tell the extent to which the plant is reliant on a xenogamous mating scheme (strictly xenogamous, highly xenogamous, etc.).

2.1.5. Testing for mixed mating systems

Many plants can set fruit both from self and cross pollen, resulting in a mixed mating system that ensures fruit- or seed-set under autogamy or xenogamy, although one or another may predominate. This test will tell us the extent to which a plant is responsive to either mating scheme.

1. Choose a given number of flower buds prior to anthesis. The number of buds may vary with availability and ease of access, but larger samples produce more reliable results.
2. Protect three fourths of these buds with pollination bags (see section 2.1.1.) and leave the other one fourth unbagged as the control. Identify each treatment with weather-resistant tags.
3. After anther dehiscence and when the stigmas are receptive, remove all bags and hand pollinate one third of the flowers

each with its own pollen (using paint brushes), one third with pollen from another flower of the same plant, and the final third with pollen from multiple plants. After that, bag the flowers again to prevent flower visitors or wind pollination. Leave bags on flowers until they are no longer receptive, then remove the bags.

4. At the end of the season, check whether any fruit developed from the bagged flowers. If all or most bagged flowers have developed into fruits, the plant species has a mixed mating system and the proportion of fruit- or seed-set obtained from the bagged treatments in comparison to the control treatment will tell whether there is a preference for self-pollination, geitonogamy or xenogamy. In the case of mixed breeding systems, honey bees can be highly effective pollinators.

2.2. Testing for pollinating agents and pollination deficit

Once one has learned about the plant mating system, it is of paramount importance to determine the agents capable of pollinating the flowers. Candidate pollinators can be abiotic (wind, water, gravity, electrostatic forces, rain) or biotic (birds, bats, insects and even mammals), but most of the time wind and insects are the major pollinators, and we will concentrate on these. It is useful to know whether a pollinating agent can meet the plant's full potential fruit-set or only a fraction of it. In the latter case, the plant may be under a pollination deficit and its fruit or seed production sub-optimal.

2.2.1. Testing for wind pollination (anemophily)

This test will tell us the extent to which a flower species is wind pollinated (*anemophilous*). It can be exclusively anemophilous in which pollinators do not contribute to fruit- or seed-set or partially anemophilous in which case pollinators can be useful for optimizing yield, examples of which include coconut, canola, olive and castor bean.

1. Choose a given number of flower buds/inflorescences prior to anthesis.
2. Protect half of these buds/inflorescences with muslin bags (mesh large enough to allow pollen grains to pass through but not insects) and leave the other half unbagged as the control. Identify each treatment with weather-resistant tags. In the case of multiple flowers on an inflorescence, a swipe of acrylic paint on the pedicel works well for identifying the treatments. The bags should remain on the flowers/inflorescences while the stigmas are receptive and can be removed afterwards.
3. To control for bag effects on wind transfer of pollen, include inside and outside bags a small sticky surface, such as a microscope slide covered in a thin coat of petroleum jelly, with which one can compare wind-borne pollen deposition in- and outside the bags. Care must be taken in interpreting results as muslin bags may reduce the level of wind pollination. Observations should be made of the wind direction and the location of the pollen source to determine if a better arrangement of plants might affect the level of wind pollination.
4. A few days later, check whether fruit has developed from the bagged flowers/inflorescences. If not, one can conclude that wind plays little or no role in pollinating that species. In the case of fruit development, the proportion of fruit- or seed-set in relation to the control treatment will tell us the degree of wind dependence by that species.
5. If hand-selfing, geitonogamy, and cross pollination treatments are also performed, one can assess for interactions of these with wind and determine optimum combinations with wind for maximizing fruit- or seed-set. To validate the cross pollination trials it is important to ensure that compatible polliniser varieties are flowering nearby.

2.2.2. Testing for biotic (honey bee) pollination – single visits

With this test, one will be able to check the role of biotic pollinators, in our case the honey bee, in fruit- or seed-setting of a particular plant species. In nature, fruit-set usually happens after repeated flower visits by one or more species of pollinator, but when evaluating different candidate pollinators, it is best to compare fruit-set on the basis of single flower visits; this is the most equitable way to compare innate pollen vectoring capacity among flower visitors. The investigator will bag unopened flowers, un-bag them after they open, observe a single visitor, re-bag the flower, then follow the flower's development for subsequent fruit or seed. The flower now has a history, and the efficacy of the specific agent can be compared with others (Vaissière et al. 1996). It is good practice to have a second flower open at the same time which can be rebagged without being visited to act as a control for bag effects as well as a set of non-manipulated and labelled flowers as open-pollinated controls. Depending on the flower species, the standing stock of nectar or pollen may build up in the bagged flower to the extent that it may influence behaviour of bees visiting newly exposed flowers. To check whether this is affecting forager behaviour, the behaviour of bees visiting previously bagged flowers can be compared to visitors to flowers that have not been bagged.

1. Choose a number of flower buds prior to anthesis.
2. Protect these buds with pollination bags (section 2.1.1.) and identify with weather-resistant tags.
3. After the flower opens remove the bags and watch for the first visit of a honey bee. Rebag the flower after the bee leaves it. The bag should remain on the flower while it is still receptive to avoid undesired visits and should be removed afterwards. Limit observations to the same time each day and to weather conditions that are suitable for insect flight.

4. The following measures may be taken at the time of bee observation and retained for possible use as explanatory covariates: length (sec) of visit, whether the bee is collecting nectar or pollen, ambient temperature, wind speed, and relative humidity.
5. At harvest, check whether fruit has developed from the visited flowers and compare fruit-setting results with those from bagged controls, hand-selfing, geitonogamy, cross pollination, and open-pollinated treatments to know the contribution of a single honey bee visit to the pollination needs of that species.
6. A modification of this method employs a direct measure of Pollinator Effectiveness after Spears (1983):

$$PEi = \frac{(Pi - Z)}{(U - Z)}$$

where Pi = mean number of seeds set per flower resulting from a single visit from pollinator i, Z = mean number of seeds set per flower receiving no visitation, and U = mean number of seeds set per flower resulting from unlimited visitation.

2.2.3. Testing for biotic (honey bee) pollination – multiple visits

Although single-visit fruit-set is a standardized measure of pollination efficiency and independent of pollinator foraging density (Spears, 1983; Sampson and Cane, 2000; Dedej and Delaplane, 2003) in flowers bearing many ovules (ex. apple, pear, melon, pumpkin and kiwi) a single honey bee visit is usually not enough to deposit all the pollen grains needed to set the fruit or to fertilize most of its ovules.

1. Choose a given number of flower buds prior to anthesis.
2. Protect these buds with pollination bags (section 2.1.1.) and identify with weather-resistant tags.
3. Randomly designate each flower as a recipient of 1, 2, 3, or 4 (or more depending on plant species) honey bee visits, remove bags after flowers open, and observe each flower for its assigned number of flower visits.
4. After the assigned number of flower visits is achieved, rebag the flower until it is no longer receptive, after which the bag is removed.
5. A few days later, check whether fruit has developed from the visited flowers and compare fruit-setting results with those from bagged and hand-self, geitonogamy, cross pollination, and open control treatments to know the importance of multiple honey bee flower visits to that particular plant species. The treatment which produces the closest fruit- or seed-set to the best hand-pollinated (or open-pollinated) treatments determines how many honey bee visits are necessary to set acceptable yields.

These can be tiring and time consuming experiments because although one can have many marked flowers within one's visual field, it is usually not possible to observe all flowers at the same time, and bees may take a long time to visit those particular unbagged flowers, especially when there are other flowers around. Some investigators get around this problem by offering freshly-cut female flowers on long extender poles to bees visiting nearby flowers in the patch (Thomson, 1981; Pérez-Balam *et al.*, 2012). This method takes some skill to avoid disturbing the natural foraging behaviour of bees and is obviously only good for destructive measures such as pollen deposition on stigmas (see section 3.2.), but it can greatly speed acquisition of data. In any case, observations should be done at roughly the same period of each day to avoid diurnal variations in flower receptivity. Also, one must not allow a different flower visitor to land on the flower while waiting for specifically honey bee visits; otherwise that flower must be discarded and all work invested on it is lost.

An alternative approach is to use a video camera that follows groups of flowers as they open. A quantitative analysis of the recording will reveal relationships between the number of visits each flower receives and its subsequent seed set. Because flowers are not enclosed, build up of pollen and nectar reflects natural rates. As the relationship between number of bee visits and seed set can only be determined if there is less than full set (once seed set is maximized additional visits are superfluous), it may be necessary to bag flowers (section 2.1.1.) after they have been videoed for an appropriate length of time to prevent full pollination. This method has the advantage that the number of visits required for full pollination can be measured directly rather than estimated as it may be when just measuring the effect of single bee visits.

2.2.4. Fruit-setting experiments at the field level

The methods listed above (sections 2.1. – 2.2.3.) are useful for determining the mating and pollination requirements of a plant and the proportion of a plant's pollen-vectoring needs met by honey bees, other visitors, wind, or self. But honey bees are commonly used as pollinators in high-density agriculture, and when designing fruit-set experiments with crops, one must be aware that cultivated plants can compensate for pollen limitation with longer flowering periods or more flowers. Similarly, fruit- or seed-set can be resource-limited. Therefore, working on the basis of individual flowers or inflorescences may over-estimate yield potentials at the basis of the crop. For these reasons, when working at the scale of agricultural production, the experimental unit should be a plot or a field, and never lower than a whole plant (Vaissière *et al.*, 2011).

Following this argument, at the field level the whole plant or plot (Fig. 5) is to be caged in the exclusion experiments, honey bee colonies are introduced into the areas where their effectiveness as a crop pollinator is to be tested, and fruit or seed production is compared to open fields with no supplemental honey bee introductions. One must also take into account the growth conditions and mating system of the target crop. For example, some crops are negatively affected by shading, others are male sterile and need the presence of male-fertile plants, and others are generally xenogamous and require a compatible cross-variety within the experimental cage.

Fig. 5. Honey bee exclusion experiment: caged plots in soybean plantation.

3. Measuring pollen on bees and pollen deposition on stigmas

Pollinator performance can be thought about with at least three organizing concepts: (1) measuring fruit- or seed-set that results after flower visitation, (2) measuring pollen load on pollinators and their pollen deposition onto stigmas, or (3) measuring plant reproductive success post-pollination, i.e. fertilization efficiency (Gross, 2005; Ne'emen *et al.,* 2010). For our purposes, we are focusing on the first two concepts because reproductive success depends not only on the amount of pollen vectored by pollinators but also on additional factors such as pollen pistil interaction and female choice (Herrero and Hormaza, 1996). In order to evaluate pollinator performance we can study the pollen carried by bees as well as the pollen effectively deposited on the stigmas.

Most of the following methods have been discussed in detail in pollination methodology books (Kearns and Inouye, 1993; Dafni *et al.*, 2005).

3.1. Identifying and evaluating pollen quantity and quality transported by bees

The first step to identify and analyse the pollen transported by bees is to remove the pollen grains from the bees' bodies. Several techniques are available for removing pollen grains from insects, usually mechanically by washing and vortexing the insect body (for example in 50% ethanol), removing the insect, precipitating the pollen grains by centrifugation, and using the pollen grains for further analyses (see Jones, 2012 for a review on pollen extraction from different insects). For studies of pollination success, the pollen packed in the corbiculae should first be removed since it is usually not available for pollination. It is sometimes possible to refine this method by only removing the pollen from the areas of the bees that have been observed to touch the stigma.

3.1.1. Microscopic pollen identification and making archival reference slides

Pollen from different plant species can usually be distinguished based on diagnostic traits such as pollen grain size, exine sculpturing and number and size of the apertures (pores or furrows). It is important to keep in mind when working with fresh pollen that the degree of pollen hydration affects external pollen appearance. Transmitted light microscopy is the most widely used technique for pollen identification using fresh, acetolyzed and stained pollen, but scanning electron microscopy (SEM) is also used to study surface details of the exine. In pollen reference collections, pollen grains are usually subjected to acetolysis that removes the protoplasm and leaves the exine (Erdtman, 1969; Kearns and Inouye, 1993). The acetolysis solution contains glacial acetic acid and concentrated sulphuric acid (9:1). According to Dafni *et al.* (2005) and Kearns and Inouye (1993) the procedure for acetolysis is as follows:

1. Add pollen sample to a solution of glacial acetic acid for 10 min.
2. Centrifuge and discard the supernatant.
3. Add a few ml of acetolysis mixture (glacial acetic acid and concentrated sulphuric acid 9:1).
4. Heat the solution gently to boiling point in a water bath, stirring continuously with a glass rod.
5. Cool the solution for a few minutes, centrifuge and discard the supernatant.
6. Resuspend in distilled water, centrifuge and decant the supernatant. Repeat this step.
7. Pollen is usually stained to increase the contrast. Several stains (such as methyl-green or fuchsin) can be used, but Safranin O is the preferred stain for most uses in palynology, staining the pollen grains pink to red depending on the amount of stain and type of pollen analysed (Jones, 2012).

After acetolysis, pollen can be preserved for further analyses or to make archival reference slides. A common procedure is to use glycerin jelly slides (Erdtman, 1969):

1. Prepare a base stock of jelly by combining 10 g gelatin, 30 ml glycerin, and 35 ml distilled water.
2. On a clean microscope slide add a drop of the prepared jelly and a sample of pollen and stain.
3. Warm the slide gently, stirring to thoroughly homogenize the mixture.
4. Add a cover slip, sealing with nail polish or other varnishes around the edges.

Identification keys and atlases with pollen images are available both in general and for specific taxa (Kearns and Inouye, 1993; http://www.geo.arizona.edu/palynology/polonweb.html).

3.1.2. Pollen identification (palynology) with molecular methods

Alternatively, pollen can be identified using molecular methods. This is straightforward if the pollen grains carried by the bees belong to just a single species since a large amount of pollen grains can be pooled for DNA, but analyses are more difficult if the bees carry a mixture of pollen grains from different species. In this case a possible approach is to use single pollen genotyping strategies (Matsuki *et al.*, 2008; Suyama, 2011) that allow PCR amplification of the genome of single pollen grains. Molecular pollen identification can be useful for assessing bee cross pollination efficacy; if a bee's corbicular pollen load contains a number of variety-specific pollens, this is evidence that the bee is foraging across pollinisers.

3.1.3. Tracking pollen identity

For some purposes in ecological research, target pollen or pollen bearing pollinia can be "tagged" to track pollinator dispersion range and pollination success. Morphological markers such as colour, size and shape polymorphisms may work as long as the work is limited to a few specific taxa. Early attempts to tag non polymorphic pollen were based on the use of radioisotopes or fluorescent dyes as pollen analogues (Dafni, 1992). With progress in genetic engineering, GFP-tagged pollen grains have also been used to track pollen identity in transgenic plants (Hudson *et al.*, 2001).

3.1.4. Pollen quantity

Several methods can be used to evaluate the number of pollen grains attached to bees.

3.1.4.1. Haemocytometers

This is the most common method for counting pollen grains. A drop of a known volume of suspension of collected pollen is placed under the microscope and the number of pollen grains counted allowing the calculation of the total number of pollen grains in the whole volume. Haemocytometers were initially developed to count blood cells, but they can also be used to count the number of pollen grains in a standard volume of liquid containing pollen (see Human *et al.*, 2013 for more information on using haemocytometers in honey bee research). The steps are as follows:

1. Collect pollen as described in section 3.1.
2. Suspend pollen grains in a known volume of 70% ethanol and vortex to assure homogenous mixing.
3. Remove a sample of pollen suspension with a pipette and place in haemocytometer.
4. View and count pollen grains under a microscope. Haemocytometer manufacturers provide the known volume of suspension under the viewing area and provide easy instructions for extrapolating object counts back to absolute counts in the original suspension (sample).
5. When the number of pollen grains is very low, a measured drop of the suspension can be placed on a lined microscope slide and all the grains counted.

3.1.4.2. Alternative methods

More expensive techniques such as electronic particle counting (Kearns and Inouye, 1993) and laser-based counters (Kawashima *et al.,* 2007) can also be used. In some situations it is possible to directly count pollen grains on a bee's body with a stereomicroscope (Nepi and Pacini, 1993).

3.1.5. Pollen viability and quality

There is disparity in results among different methods for appraising pollen viability and quality; for this reason the most robust approach is to use a combination of methods, such as those provided below, that allow a more precise estimation of pollen viability and quality (Dafni and Firmage, 2000).

3.1.5.1. Pollen viability

One approach is to evaluate viability prior to germination. The most common test is the fluorochromatic reaction (FCR test) based in fluorescein diacetate (FDA) (Heslop-Harrison and Heslop-Harrison, 1970; Pinillos and Cuevas, 2008). This test evaluates the integrity of the plasmalemma of the pollen vegetative cell and activity of nonspecific esterases of the cytoplasm, and only viable pollen grains will fluoresce under the microscope (Fig. 6). Another commonly used viability test is Alexander staining (Alexander, 1969); viable pollen stains crimson red while aborted pollen stains green. Additional tests such as the use of tetrazolium dyes, X-Gal, isatin or Baker's reagent can also be used (see Dafni and Firmage, 2000 for a review). However, in most viability tests not all the viable pollen grains are able to germinate, and consequently the percentage of pollen germination is usually lower than the percentage of viable pollen.

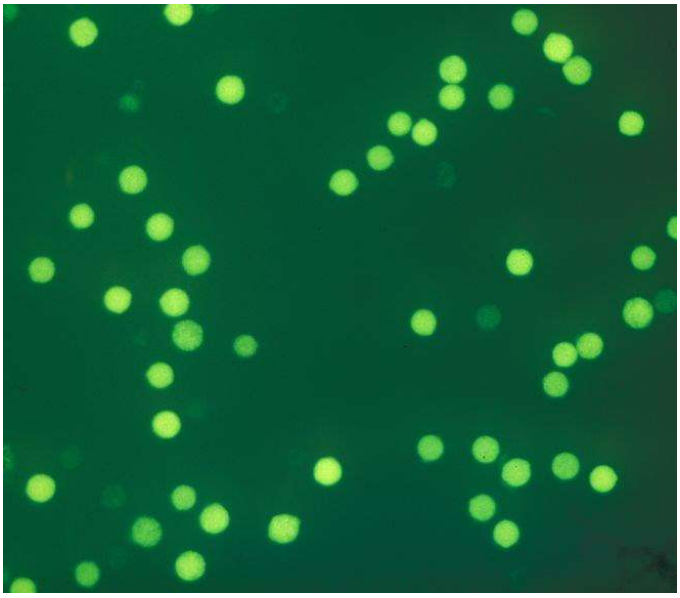

Fig. 6. Pistachio pollen stained with FDA: viable pollen grains show bright fluorescence compared to non-viable pollen grains.

***3.1.5.2. Pollen germination and pollen tube growth* in vitro**

The evaluation of pollen germination *in vitro* takes into account not only pollen viability but also pollen vigour (Shivanna and Johri, 1985; Shivanna *et al.*, 1991). One drawback of the method is that the germination medium and germination conditions (e.g. pollen pre-hydration, temperature conditions) must be optimized for each species to avoid false negatives. Different media for *in vitro* pollen germination have been recommended for several species (Taylor and Hepler, 1997), mainly using the basic medium developed by Brewbaker and Kwack (1963) in a sucrose solution with or without agar, depending on the species. The optimum method has to be tested for each species empirically. A pollen grain is considered as germinated when the length of the pollen tube is at least twice the diameter of the pollen grain.

3.2. Evaluating pollen identity, quantity and quality on stigmas

For some studies, mainly in the field, it is necessary to avoid contaminating stigmas with non-target pollen. This means it is necessary to prevent bees from visiting target flowers. Different strategies have been used (Kearns and Inouye, 1993), including a variety of tubes and capsules for small flowers, plastic pieces to cover just the pistils, or nylon or paper bags to enclose the flowers, the inflorescences, or whole plants before the experiment begins. Errors in flower sampling can be minimized by removing all opened flowers before the experiment begins. The main disadvantage of these enclosures is that the microenvironment in the flower (mainly temperature and humidity) can be altered and depending on the experiment this can have implications for the results. In any case, air-permeable mesh or net bags are likely to have a smaller effect on flower microenvironment than paper bags or plastic enclosures. In some cases, emasculation might be needed to avoid self-pollination, although emasculation can affect subsequent pollinator behaviour. The possibility of emasculating is dependent on the morphology of the flower and should be carried out carefully, especially if dealing with small flowers to avoid accidental self-pollination or damage to the flower (Hedhly *et al.*, 2009).

3.2.1. Identifying pollen on stigmas

Identifying pollen deposited on stigmas can provide evidence of the percentage conspecific pollen deposited and the likelihood of stigmas being clogged by pollen from other species. This constitutes perhaps the most unambiguous and precise measure of pollination success, *sensu stricto*. The stigmas can be collected and washed in 70% ethanol and the pollen grains released can be observed using similar procedures to those described for identifying pollen on bees (see section 3.1.). Molecular markers have also been used to identify pollen deposited on the stigma (Hasegawa *et al.*, 2009).

3.2.2. Quantifying pollen deposited on a receptive stigma per visit or unit time

Usually this parameter is measured by counting the number of pollen grains deposited on the stigma per visit or unit time, regardless of fertilization success. This method implies the microscopic examination of pollen germination and tube growth in the stigma and style. Different stains that stain pollen grains differentially from the surrounding stigmatic tissues can be used. Usually the stigma is gently squashed under a coverslip after staining to better visualize the pollen grains. If needed, stigmas can be fixed in FAA (formaldehyde - acetic acid - 70% ethanol [1:1:18]), 4% paraformaldehyde, glutaraldehyde (2.5% glutaraldehyde in 0.03 mol/L phosphate buffer), 3:1 (v/v) ethanol – acetic acid, or just 70% ethanol and stored at 4ºC for later examination. However, it should be taken into account that before germination, pollen needs to adhere to the stigma and hydrate. Fixing can remove non-adhered pollen grains and consequently the estimate of pollen load may be lower than if fresh stigmas were analysed. In some cases, softening the fixed stigmas should be performed before staining and squashing; this can be done by autoclaving the samples at 1 kg / cm^2 for 10 to 20 min in 5% (w/v) sodium sulphite and rinsing in distilled water or, alternatively, 1M NaOH can be used for 1 h following a rinse in distilled water. Each of the following methods is acceptable for determining number of pollen grains and extent of their germination.

1. Epifluorescence microscopy. This is the most widely used method for visualizing pollen tubes. Stain the pollen grains and pollen tubes with aniline blue (specific for callose, a polysaccharide present in pollen tube walls and plugs produced in pollen tubes of most Angiosperms) (Fig. 7) and observe under fluorescence microscopy. The usual mix is 0.1% (v/v) aniline blue in 0.3 M K3PO4 (Linskens and Esser, 1957). The observer can directly determine the percentage pollen germination on the stigma.
2. Light microscopy. Different methods are available that do not require epifluorescence:
   a. Methyl green and Phloxine B (Dafni *et al.,* 2005). Non-germinating grains stain dark brown-red, whereas in germinating pollen grains, the empty grains stain green and the pollen tubes red.
   b. Stain with 1% basic fuchsin: 1% fast green (4:1) (Kearns and Inouye, 1993). De-stain and soften the tissue in lactic acid for 12 hours and then squash the tissue under a coverslip. Pollen tubes stain maroon and the background remains white.
   c. Acetocarmine/basic fuchsin (Kearns and Inouye, 1993). Add a drop of acetocarmine, followed by a drop of 3% aqueous basic fuchsin and de-stain with a drop of absolute ethanol. Pollen cytoplasm stains red.
3. Scanning electron microscopy can be used to visualize the whole stigmatic surface, but this is a more time-consuming and complicated than light microscopy.

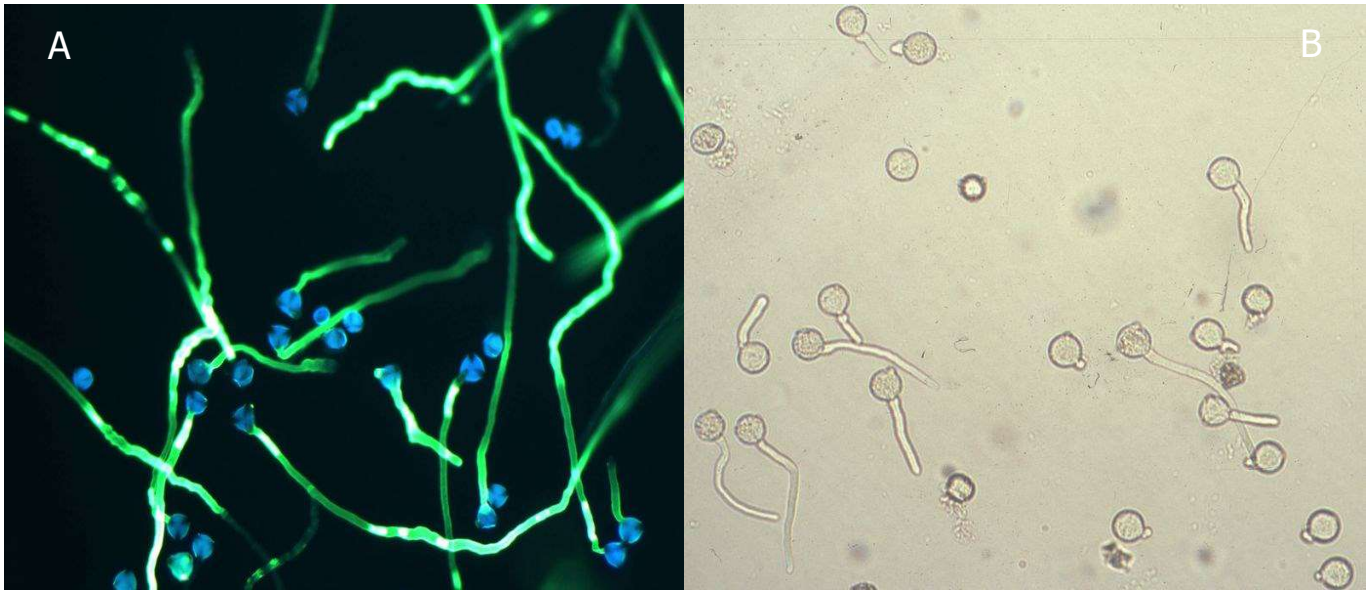

Fig. 7. Pollen germination *in vitro*. Left (A): pollen from Japanese plum stained with aniline blue. Right (B): pollen from sweet cherry, unstained.

3.2.3. Evaluating stigmatic receptivity

Pollination success is dependent on stigmatic receptivity since only insect visits to receptive stigmas can be considered as effective pollination visits. A receptive stigma allows pollen grain adhesion that can be followed by pollen hydration and germination. Stigmatic receptivity can be evaluated directly by studying conspecific pollen germination (see 3.2.4.) or indirectly by studying activity of enzymes (Dafni *et al.*, 2005) such as esterase (with a benzidine solution) or peroxidase (with alpha-naphthyl acetate) or the presence of exudates in wet stigmas stained with Sudan black or auramine O (Kearns and Inouye, 1993). Herein, we discuss determining stigmatic receptivity only by studying conspecific pollen germination.

3.2.4. Evaluating pollen germination and pollen tube growth *in vivo*

The most common procedure is to study pollinated stigmas and styles in squashed preparations stained with aniline blue (specific for callose) and observed under fluorescence microscopy (as described in section 3.1.). The tissues have to be softened, usually in 5% sodium sulfite to allow squashing, and the time of softening is species dependent; it is advisable to start processing the samples overnight and, if needed, the samples can be autoclaved at 1 kg/cm^2 for 10 min in 5% (w/v) sodium sulphite or placed in 1M NaOH for 1 h. Depending on the species, varying concentration of sodium hydroxide (from 1N to 4N) at different temperatures (ambient temperature overnight or 60ºC for an hour) can also be tried for softening. For staining, 0.1% (v/v) aniline blue in 0.3 M K3PO4 (Linskens and Esser, 1957) can be used and the observations made with a fluorescence microscope. Pollen tube walls and the callose plugs produced by growing pollen tubes show a distinct fluorescence signal (Kearns and Inouye, 1993). This test is also useful to determine the presence of gametophytic self-incompatibility in which incompatible pollen tubes get arrested during pollen tube growth in the style.

4. Measures of colony level pollination efficacy

The foraging activity of social insects is in part controlled by their colony and its requirements. Honey bees are usually managed at the colony level for pollination, i.e. colonies may be subject to particular management practices to influence their foragers' pollinating activity.

This section covers measures for assessing the pollination efficiency of honey bees at the colony level. To determine a colony's pollination efficiency, it is usually necessary to have an understanding of the efficiency of individual foragers at pollinating flowers from the crop to determine which assessments should be used. For example, for a crop like kiwi that only produces pollen, a measure of kiwi pollen collected by a colony will indicate the portion of foragers that are visiting the crop. Likewise for avocadoes and hybrid carrots it is only nectar foragers that are carrying out pollination activities and pollen foragers will be of less interest.

The measures of colony performance generally fit into two types. Measures can be made on returning foragers which generally relate to what flowers the foragers are visiting, whether they were foraging for pollen or nectar, and for some crops how effective they were at pollinating the crop. The second approach is to study the behaviour of foragers in the field and then attempt to determine which colony they came from. These types of studies are often carried out to assess the effect of colony level manipulations designed to increase the number of honey bees visiting a crop or their behaviour when they are visiting the crop, e.g. to measure the effect of:

1. the timing of colony introductions,
2. placement of colonies in the crop,
3. colony strength (number of bees and amount of brood),
4. organization of the colony, e.g. location of the brood or proportion of the brood that is uncapped,

5. practices to change foraging behaviour, e.g. feeding pollen or syrup and using pollen traps, and
6. competing floral sources.

This section covers methods for assessing

- pollen foraging
- nectar foraging
- colony foraging rates
- fraction of side working behaviour
- relationship between foragers and their hives

4.1. Proportion of foragers from a colony visiting a crop

4.1.1. Pollen trapping

The number of foragers from a honey bee colony that is collecting pollen can be estimated using pollen traps (Goodwin, 1997; see Human *et al.*, 2013 for more details). This is particularly relevant if the crop of interest only produces pollen (kiwi) or if pollen foragers are more efficient pollinators than nectar foragers (apple, almond) because they have a greater likelihood of contacting the stigma. Pollen traps are devices with grids (Fig. 8) that fit across the entrance of a hive. With some designs, the hive entrance is blocked and the trap forms a new entrance. Returning foragers must walk through the grid to enter their hive. Bees prefer not to walk through pollen traps if they can avoid it and will use any other gaps in a hive body as an entrance once a pollen trap is fitted. These holes need to be blocked to ensure that all bees are using the pollen trap. It is worth checking the hives several days after the trap is fitted to make sure all bees are entering and leaving the hive through the pollen trap.

As returning bees carry pollen through the trap the grid scrapes some of the pollen pellets from their corbicula. The pellets then fall into a tray where they can be collected. The proportion of pollen pellets removed depends on the size and shape of the holes in the grid and the size of the pollen pellets the bees are carrying. Pollen pellet size, and consequently the efficiency of a pollen trap may vary with both the plant species, the time of day the pollen is collected, and meteorological conditions (Synge, 1947). The inside of a pollen trap can become blocked over time. Depending on the design of the trap it may be difficult for hive cleaning bees to carry dead bees through the trap and for drones to move through it. When these accumulate on the inside of the trap it can reduce the ease with which foragers move through the trap and hence the foraging ability of the colony. If traps are to be used for extended periods of time they should be checked regularly for blockages.

4.1.1.1. Determining pollen trap efficiency

Pollen traps are variable in their design, so it is advisable to determine the efficiency of a trap at collecting the pollen. This can be achieved by counting the number of bees entering the trap carrying the pollen pellets of interest and determining the percentage that are collected in an empty pollen trap drawer (Levin and Loper, 1984; Goodwin and Perry, 1992).

Fig. 8. Grid on a pollen trap.

4.1.1.2. Number of pollen traps

Most studies will require data to be collected from a number of colonies as there can be large differences in the plant species neighbouring colonies are visiting. The level of replication required will depend on the amount of variation between colonies and the size of the difference to be detected. Balancing colonies with regard to the amount of brood and number of bees they contain (see Delaplane *et al.*, 2013) will reduce the amount of variation in the total amount of the pollen collected.

4.1.1.3. Analysing pollen trap contents

Pollen pellets from many plant species can be identified by colour (Fig. 9). To establish the colour of the pellets of interest it is best to catch bees with pollen pellets from the crops of interest and remove their pellets so they can then be used as standards for comparing with pellets in the tray of the pollen trap. It is important to note that the colour of pellets may change depending on the light by which they are viewed and when they dry (Kirk, 2006). Because the colours of pellets from some plant species are sometimes similar, it is often necessary to measure the size of the pollen grains making up the pollen pellet and study the pollen grains' surface features microscopically to establish whether the trap contents can be sorted by eye (see section 3.1.1.).

Because of the large amounts of pollen that may be trapped at times, it may be necessary to subsample the pollen trap contents. The contents of a trap are often layered as bees collect pollen from different plant species at different times of day. It is therefore necessary to mix the contents of the trap thoroughly before subsampling. If determining the weight of different pollens trapped rather than the number of pellets trapped, it is necessary to first dry

Fig. 9. Pollen pellets.

samples to a constant weight as their moisture content may vary between species, time of day, and between days. Samples of pollen pellets can be stored for short periods of time at room temperature. However they may eventually develop mould making them difficult to analyse. It is therefore good practice to freeze samples if they are not going to be analysed at the time of collection.

4.1.1.4. Effect of pollen traps on foraging

Honey bees losing pollen pellets while moving though a trap still go through their normal behavioural repertoire associated with scraping the pollen off their legs into a cell (McDonald, 1968). If using high efficiency pollen traps, it is important to note that they may reduce brood rearing after prolonged use (Eckert, 1942) and cause colonies to increase pollen collection (Levin and Loper, 1984). For this reason, pollen trapping with high efficiency traps should never extend beyond a few days to prevent compromising colony strength.

4.1.2. Nectar collecting

For some crops it is possible to estimate the number of visits that bees from a colony make to collect nectar. This is important for crops were nectar foragers are the most important pollinators (avocadoes, hybrid carrots) This can sometimes be achieved by a chemical analysis of the stored honey if the nectar from the plant of interest has a unique chemical profile e.g. avocados (Dag *et al.*, 2006). It can also sometimes be achieved by an analysis of the pollen contained in the honey; however, care must be taken in interpreting results as pollen grains from some plant species are more likely to be present in honey than other species. Also, the amount of honey produced is not only affected by the amount of nectar collected by a colony but also the amount consumed.

4.1.3. Proportion of colony bees collecting pollen

For many flowers that are visited by both pollen and nectar foragers, pollen foragers are better pollinators (Free, 1966). Because of the close proximity of anthers and stigmas, pollen foragers are more likely than nectar foragers to touch both structures. Some bees collect both pollen and nectar on a foraging trip. The proportion of pollen and nectar foragers can be determined by observing the behaviour of foraging bees. Nectar gathers will probe the base of the petals while pollen foragers usually scrabble over the anthers.

4.1.4. Colony foraging rate

A colony's foraging rate refers to the number of foraging trips a colony makes during a day. Generally the more foraging trips bees from a colony make to a crop, the more effective the colony will be at pollinating the crop. The number of bees foraging from a colony can be estimated by counting bees entering the hive (Baker and Jay, 1974). This is usually easier than counting bees leaving a hive as returning foragers approach more slowly. When counting the number of returning bees over a set length of time it is important to do this without disturbing the returning bees. The presence of an observer at the front of a hive may confuse bees and delay their return. This can be avoided by using a hide that can be left in front of the hive. Alternatively, a video camera will be less obtrusive and can be left in position for the bees to become accustomed to it. Video has the advantage that allows the action to be observed in slow motion. The data are reported as returning bees per minute.

Depending on the questions being answered the physical counts or video data may need to be backed up with samples of returning bees. Honey bees observed returning with pollen must have been foraging, however honey bees returning without pollen might be nectar foragers or bees going on orientation flights. Returning bees can be captured by blocking the hive entrance and allowing the returning bees to collect on the outside. The bees without pollen can then be captured. Dissecting the bees and measuring their crop weight will differentiate bees that were on orientation flights from bees that were foraging for nectar.

4.1.5. Fraction of bees side-working flowers

On some flower species (almonds, apples) the flower architecture allows bees to approach the nectaries by climbing through the anthers past the stigma (top working bees, Fig. 10) or from the side of the flowers where the push their tongues between the base of the anthers (side working bees, Fig. 11). Side working bees are less likely to touch the stigma than top working bees. The proportion of bees carrying out these behaviours varies with flower architecture and with the experience of the bees. The data are collected by modifying the methods given in section 5.1. to report number of top working, or side working, bees for a given number of flowers or measured area of crop.

Fig. 10. A top-working honey bee visiting an apple flower.

Fig. 11. A side-working honey bee visiting an apple flower.

4.2. Relationship between foragers and their hives

It may be necessary to determine whether the bees in a part of a crop are coming from a particular hive or hives. This might be of interest when manipulations of colonies are carried out to alter honey bee foraging behaviour that cannot be detected by studying foragers as they return to their hive. It might also be used to determine where in a crop bees from particular colonies are foraging. There are several methods of achieving this.

4.2.1. Marking bees in the crop

This can be achieved by catching bees on the crop, marking them, and opening hives in the evening and searching for marked bees. Bees will usually need to be immobilized before marking. This can be done by chilling the foragers, anoxiating them with CO_2 or anesthetizing them with chloroform. Care needs to be taken when choosing to use CO_2 as it has been reported to inhibit pollen collection (Ribbands, 1950, Brito *et al.*, 2010).

Acrylic paints can be used to mark bees if they only need to be marked for a single day as the paint may wear off after this time. Acetone based paints will last longer. By using a range of colours and positions of spots on the thorax and abdomen it is possible to individually and distinctively mark large numbers of bees. An alternative, more costly method, is to use purpose-made plastic queen tags glued on the thorax of worker honey bees (Fig. 12). As there are often a large number of bee colonies foraging from a crop, it is usually necessary to mark large numbers of bees to obtain adequate recovery rates.

Colour-coded ferrous tags glued to the thoracic dorsum of an individual forager in the field can be retrieved at the hive entrance with magnets (Gary, 1971). This technique enables studies of spatial distribution of bees in an area.

4.2.2. Marking bees according to their hives

Strains of bees with the visible mutation *cordovan* can be used as it is possible to identify these workers in the field (Gary *et al.*, 1981). The bees in a colony can also be fed with radioactive elements (Levin, 1960) or made to walk through a marking block fitted to the entrance of a hive that marks them with coloured dye (Howpage *et al.*, 1998). Workers marked with any of these methods can be searched for in the field.

5. Determining crop-specific recommended pollinator densities

An aim of some research in agricultural pollination is to provide guidelines for stocking honey bee colonies in a crop to maximize pollination in the most economical way. There are two general approaches to designing experiments for determining optimum stocking densities: (1) indirect extrapolations from densities of foraging bees observed in small plots, along transects away from colonies, or in cages; or (2) direct tests of colony densities on whole fields.

Fig. 12. Foragers with queen tags.

5.1. Indirect extrapolations

The stocking rates of colonies required can be estimated indirectly by using the pollination potential of individual foragers (i.e. seed set per flower visit; sections 2.2.2. and 2.2.3.) and extrapolating the number of foraging honey bees and colonies required to pollinate a crop (Goodwin *et al.*, 2011). This presupposes our ability to reliably measure bee densities in the crop.

5.1.1. Bee densities in small field plots

Appropriate bee density measures vary according to plant growth habit, planting arrangement and conformation of flowering. Examples of density measures that may be used include:

- Bees per m^2 or larger area (especially for vine crops)
- Bees per tree (tree fruits and nuts)
- Bees per flower or larger number of flowers (berries, vine crops, cotton, sunflower)

Considerations when counting bees:

- Make several observations through the duration of the flowering period
- Subsample within a day, recording time of samples as local solar time
- Sample during weather conditions that are favourable for foraging, i.e., temperature ≥ 15°C, wind < 16 km/h, no rain (flowers dry) and preferably sunny
- Also sample for pollinator diversity

Considerations in choosing sampling sites:

- Use multiple sampling sites within a field
- Choose representative sites that are at least 5 m from the field edge
- Use > 1 m of row in row crops
- Use plots of > 1 m^2 in broadcast-seeded plots and non-row crops
- Use individual branches of trees in orchards
- Use individual flowers if large enough (e.g., sunflowers)

Considerations when measuring yield are as in sections 2 and 6.

Vaissière *et al.* (2011) give detailed suggestions about assessing pollination needs of different types of crops. They also provide useful data collection sheets for recording bee count and yield information for crop of different growth habits.

5.1.2. Field-scale transects

Colonies can be placed at one end of a crop field, a linear sampling transect established across the field, and the number of bees visiting flowers counted (visits per min) and seed-set assessed at points along the transect to determine whether these bee visitation and yield decline with distance from the colonies (e.g. Manning and Boland, 2000). This may only be useful in large areas of crops. It is good practice to repeat the trial with the colonies at the other end of the crop. For transects,

- Use very long fields (> 400 m)
- Establish a bee density gradient by locating colonies at one end of the field only
- Use multiple sampling sites at intervals of 100-200 m away from the colonies

5.1.3. Cage visitation rates

Observations on visits to flowers of a crop are sometimes made on plants within cages where bee densities can be controlled and replicated more easily than in fields (e.g. Dedej and Delaplane, 2003); cages often are ca. 2 m^3 and constructed of Lumite® (e.g. BioQuip Products; Rancho Dominguez, CA, USA). These tests are useful for gaining insight about the relative impact of bee visitation on the yield response of a crop. The artificial environment within a cage, however, can affect both bee behaviour and plant growth, and this further limits the ability to transfer findings about effects of bee density on crop yield to recommendations about colony stocking rates in normal field situations. Thus cage tests provide only a very general idea of comparative usefulness of different colony densities and are much less useful than studies using small plots or whole fields.

5.2. Direct tests of whole fields to find the required number of colonies per hectare

A less common approach is to stock fields with different numbers of colonies and establish whether the rates used have an effect on pollination (Palmer-Jones and Clinch, 1974; Vaissière, 1991; Brault *et al.*, 1995). Direct comparisons of different colony densities are rare because it is difficult to obtain acceptably large numbers of replicate fields for each treatment. Past research usually involved a few fields (e.g. Eischen and Underwood, 1991), or a few fields repeated over a few years with treatments rotated among fields (e.g. Stern *et al.*, 2004).

If multiple fields are available for testing, they should be:

- As similar as possible regarding cultural practices (e.g. irrigation, drainage, fertilization, pest and weed control), available pollinisers, soil type and surrounding habitat
- Far enough apart (ideally > 3 km) to isolate bee populations

If multiple fields are available, similar fields should be paired and honey bee colonies introduced into half of the fields while the other fields serve as controls without supplemental bees. A recent recommendation (Vassière *et al.,* 2011) is to use ≥ 5 fields per treatment, with bees introduced at the onset of effective flowering (i.e. at the time of first bloom that would lead to a product).

Considerations when collecting data about pollination outcome include the following:

- Use units of yield per field, plot, plant or flower as appropriate for the crop. Yield may include fruit and seed quantity and quality (see section 6).
- Alternatively, use pollen deposition (see section 3.2.), or fruit- or seed-set (see section 2). It is useful to measure pollination outcomes prior to harvest to prevent losing fruit to events

(e.g. natural herbivory, violent weather) that can confound treatment effects. Note, however, that pre-harvest measures of immature fruits do not reflect outcomes typical of agricultural commerce.

- It is advisable to estimate realized densities of bee foragers in fields resulting from the different numbers of colonies (see section 5.1.1.).

5.3. Appraising risk of competition between plants for pollination

If the target crop blooms in synchrony with neighbouring weeds or crops, there is a risk that it will not be well serviced by a limited pool of pollinators. This underscores the need to monitor forager density on the target crop. It may be useful to gauge the distribution of bees among competing plants species; see the methods outlined in section 4.1. for possible approaches. The most convenient technique may be to use pollen traps to measure the relative proportions of pollen income from different forage sources if bees are collecting pollen from all sources.

5.4. A cautionary note about recommendations

Delaplane and Mayer (2000) list recommended colony densities and their average for many crops is based on information collected from standard pollination references and historical extension bulletins. Recommendations about the optimal number of colonies per hectare ultimately often come from experiences of growers and beekeepers who have adjusted bee densities based on trial and error over time. A commonly recommended starting density is 2.5 colonies of standard strength (often cited as having ≥ 8 combs, two-thirds covered with adult bees or ≥ 6 combs well covered with brood) per hectare. This may be adjusted knowing the relationship of crop yield with factors that affect foraging activity or pollination. Examples of such factors among include:

- Plant reproductive biology, including cultivars that are more difficult to pollinate effectively, e.g. 'Delicious' apples (*Malus domestica*), because of a high frequency of sideworking honey bees; more bees are needed for such plants.
- Field size: larger fields usually need more supplemental pollinators than small fields because small fields often have greater densities of native pollinators.
- Prevailing weather: a region or season with historically poor weather for bee flight may warrant a higher stocking density.
- Competition: the extent to which the target crop is competing with weeds or neighbouring crops for a limited pool of pollinators
- Ambient densities of native pollinators

A case study of how these particular factors have been used to adjust pollination management involves lowbush blueberry (*Vaccinium angustifolium* Aiton and *V. myrtilloides* Michx.) in the northeastern USA. The crop is difficult because it requires much pollen movement, honey bees do not "buzz pollinate" (sonicate) the ericaceous flowers, many commercial fields are large and have insufficient densities of native pollinators, weather during bloom is often poor, and bees seek pollen from sources other than blueberries. Large-scale commercial growers have tested the value of increased stocking densities. Through this experience the largest growers now prefer to rent colonies that are more populous than average and stock them typically at 10-12/ hectare and up to 20-25/hectare in historically high-yielding areas (Danka: unpub. obs.).

6. Measuring harvest and post-harvest effects of pollination

The economic impact of pollinators on agricultural output transcends simple yield measures and extends into harvest and post-harvest effects as well (Bommarco *et al*., 2012; Dag *et al.,* 2007; Gaaliche *et al*., 2011). These include things like fruit sweetness, shape, weight, texture, and other flavour metrics (Gallai *et al.,* 2009). Many of these quality criteria are affected by seed number which in turn is a result of pollination efficiency (Dag and Mizrahi, 2005; Dag *et al.,* 2007). However, even when fruit have only one seed, pollination can affect fruit-quality parameters since fruit resulting from cross-pollination might differ from those stemming from self-pollination, as has been reported in mango (Dag *et al*., 1999), avocado (Degani *et al.,* 1990) and other crops. In this section, we describe major quality criteria and provide methods for their quantification. The presented protocols for assessing fruit quality are based on Kader (2002). For each crop, researchers need to define which parameters are relevant in the context of pollination efficiency.

6.1. Visual appearance

- Size: Fruit size can be measured with a sizing ring or calipers (Fig. 13). There is generally a good correlation between size and weight; size can also be expressed as number of units of a commodity per unit weight. Volume can be determined by water displacement or by calculating from measured dimensions.
- Shape: Ratios of dimensions, such as diameter-to-depth ratio, are used as indices of fruit shape (e.g. sweet pepper: Dag *et al.,* 2007) (Fig. 14).
- Colour: The uniformity and intensity of colour are important visual qualities, as is light reflectance which can be measured by any number of dedicated meters. These devices measure colour on the basis of amount of light reflected from the surface of the fruit; examples include Minolta Colorimeter,

Gardner, and Hunter Difference Meters. Internal colour and various internal disorders can be detected with light transmission meters. These devices measure light transmitted through the fruit. Fruit colour can be evaluated on the basis of pigment content, usually a function of quantity of chlorophylls, carotenoids and flavonoids.

- Defects (Fig. 15): Incidence and severity of internal and external defects can be evaluated on a five-point subjective scale (1 = none, 2 = slight, 3 = moderate, 4 = severe, 5 = extreme). To reduce variability among evaluators, detailed descriptions and photographs may be used as guides in scoring a given defect. An objective evaluation of external defects using computer-aided vision techniques appears promising. Internal defects can be evaluated by non-destructive techniques, such as light transmission and absorption characteristics of the fruit, sonic and vibration techniques associated with mass density, and nuclear magnetic resonance imaging.

Fig. 13. Measuring guava fruit size using digital calliper.

Fig. 14. Left: large sweet pepper fruit from honey bee-pollinated greenhouse. Right: small fruit from a control, unpollinated greenhouse.

Fig. 15. Right: misshapen strawberries due to poor pollination. Left: regularly and well-shaped fruits resulting from satisfactory pollination.

6.2. Textural quality

- Yielding quality (firmness/softness): Hand-held testers can be used to determine penetration force. One example is the Magness-Taylor Pressure Tester. The plunger (tip) size used depends on the fruit and varies between 3 and 11 mm. Stand-mounted testers can determine penetration force with more consistent punch speed, one example being the UC Fruit Firmness Tester. Contractual laboratory testing is available for appraising fruit firmness with instruments such as the Instron Universal Testing machine, the Texture Testing system or by measuring fruit deformation using a Deformation Tester.
- Fibrosity and toughness: Shear force can be determined with an Instron or Texture Testing system. Resistance to cutting can be determined using a Fibrometer. Fibre or lignin content can be determined by contractual lab services with various chemical analyses.
- Succulence and juiciness: Any number of commercially available fruit refractometers can be used to measure water content - an indicator of succulence or turgidity.

6.3. Flavour

- Sweetness: Sugar content can be determined by chemical analysis for total and reducing sugars or for individual sugars (e.g. yellow pitaya: Dag and Mizrahi, 2005). Total soluble solids can be used as a proxy measure of sugar content because sugars are the predominant component of fruit juice soluble solids. This parameter is measured with a fruit refractometer.
- Sourness (acidity): pH of extracted juice can be quantified with a pH meter or pH indicator paper. Total titratable acidity can be derived by titrating a specific volume of the extracted juice with 0.1 M NaOH to pH 8.1, then calculating titratable acidity as citric, malic, or tartaric acid, depending on which organic acid is dominant in the commodity.
- Astringency is quantified by taste test or by measuring the solubility of tannin or its degree of polymerization.

- Bitterness is quantified by taste test or by measuring alkaloids or the specific glucosides responsible for bitter taste.
- Aroma (odour) is quantified by use of a human sensory panel in combination with identifying the specific volatile components responsible for the aroma of the fruit.
- A comprehensive sensory evaluation can be used to characterize the combined sensory characteristics (sweetness, sourness, astringency, bitterness, overall flavour intensity) of the fruit.

6.4. Nutritional value

Various analytical methods are available to determine total carbohydrates, dietary fibre, proteins and individual amino acids, lipids and individual fatty acids, and vitamins and minerals in fruits and vegetables. For the most part, these kinds of analyses are specialized and require the collaboration of appropriate expertise.

7. Managing bee colonies for optimum pollination

7.1. Bee attractants

Bee attractants are designed to attract bees to crops. There are several approaches to testing their effectiveness, including measuring changes in the numbers of bees visiting flowers and changes in the levels of pollination (Ellis and Delaplane, 2009). The criteria in section 2.2.4. apply, and experimental units should never drop below the level of field plot. The complicating factor in these types of trials is achieving suitable replication and controls because attractants require large areas of a crop to be treated. For this reason, the expedient of using cages is not applicable to attractant studies. Treated and control plots must have large separations between them; otherwise there is risk that if the attractant works it may draw bees away from the control plots, thus artificially enhancing the effect of the attractant. Dependent variables can be collected as described in sections 4.1., 5.2., and 5.3.

7.2. Feeding colonies

7.2.1. Feeding syrup

Colonies can be fed liquid sugar syrup (sucrose) to cause them to increase the amount of pollen they collect (Goodwin, 1997) (Fig. 16). The syrup needs to be fed inside their hives, and the container needs to include flotation to minimize bee death from drowning. Feeding an average of 1 litre of syrup, with between 45 and 65% sucrose concentration, every day has been reported to result in significant increases in pollen collection (Goodwin and TenHouten, 1991). It is important that any syrup that has started to ferment in the feeder is discarded before more syrup is added. Feeding colonies outside their hives is unlikely to cause colonies to collect pollen.

7.2.2. Feeding pollen

Colonies can also be fed pollen or pollen substitutes to promote colony growth. Feeding pollen has, however, been reported to decrease the amount of pollen a colony collects (Free and Williams, 1971). This effect does not happen in all cases (Goodwin *et al.*, 1994).

7.2.3. Testing effects of feeding regimens on pollination performance

Given that colony nutrient state can affect its performance as a pollinator, feeding regimens can be used as experimental treatments in pollination studies. In these cases the criteria in section 2.2.4 apply. Experimental units should be no smaller than a field plot and sufficiently isolated to prevent bees drifting and confounding treatments. If space is limiting, cages can be used to contain sufficient plants with colonies assigned the different treatments. Dependent variables can be collected as described in sections 4.1., 5.2., and 5.3.

7.3. Distribution of colonies within the crop

The distribution of colonies within a crop is often a controversial issue between growers and beekeepers. This is because it is easiest for the beekeeper to drop off hives in single or several large groups. However, many growers want the hives spread evenly throughout the crop in the hope that there will be an equitable distribution of pollinators. Thus, testing for colony distributions that are optimum between these competing interests may be useful. The criteria given in section 2.2.4. apply, and experimental units for different hive distribution scenarios should be no smaller than a field plot and sufficiently isolated to

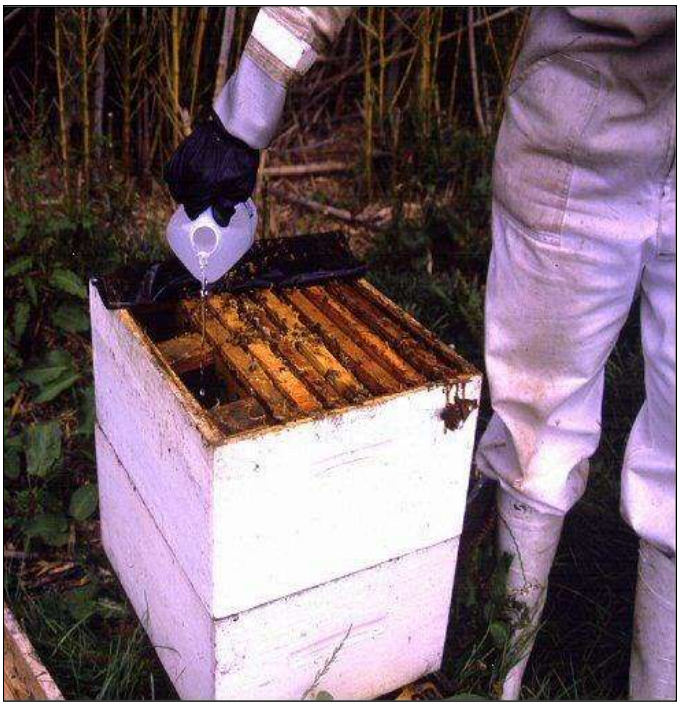

Fig. 16. Feeding a colony sugar syrup.

prevent bees drifting and confounding treatments. Dependent variables can be collected as described in sections 4.1, 5.2, and 5.3, and reported as treatment means with plot or field as experimental unit. Depending on the hive distribution patterns selected, the use of field transects may be useful as covariates or supplemental information to understand pollinator performance relative to a point source of bees. Section 5.1.2. gives useful guidance on the use of field transects.

8. Conducting pollination research in greenhouses and tunnels

Many high-value cash crops which were once cultivated exclusively in open fields are now grown in greenhouses and net-houses. This shift has been made mainly to protect plants from pests, enable out-of-season production, isolate plants for production of pure seeds, or to limit other environmental hazards (Fig. 17).

Assessing pollination activity in an enclosure is similar to assessing it in the open field (see sections 4 and 5). However, conducting pollination research in this specialized environment requires special considerations, covered below.

8.1. Carbon dioxide (CO_2) level

CO_2 enrichment has been used for many years in greenhouses to increase crop growth and yield. Since nectar production may be related to photosynthesis level, we might expect an increase in floral rewards in greenhouses with enriched CO_2 (Dag and Eisikowitch, 2000). On the other hand, if the greenhouse is completely closed and contains high biomass, intensive photosynthesis may lead to a reduction in CO_2 to lower than ambient (350 ppm) levels. There are different sensors available on the market to assess CO_2 level. Sensors that use non-dispersive infrared (NDIR) technology to measure CO_2 concentration in the greenhouse air are common and generally reliable. The sensor is placed near the leaf, i.e. the organ most affected by CO_2 levels.

Fig. 17. Bee hive placed for greenhouse melon pollination.

8.2. Solar radiation

In recent years, various modifications have been made to the spectral characteristics of greenhouse covers. These alterations are designed to protect greenhouse-grown crops from herbivorous insects and insect-borne viral diseases and to suppress proliferation of foliar diseases. These goals are achieved through partial or complete absorption of solar UV radiation (Raviv and Antigunus, 2004). However, UV radiation is also essential for honey bee and bumble bee navigation (Sakura *et al.,* 2012). Use of UV-absorbing sheets as well as UV-opaque covers (such Perspex and fiberglass) can therefore be damaging to bees' pollination activity in enclosures (Dag, 2008). The degree of UV absorption can be evaluated using UV sensors (10–400 nm), and opacity to UV radiation can be assessed by observing the shadow of an object in the enclosure through a UV filter; if a distinct shadow cannot be seen, then the UV radiation is diffuse.

8.3. Temperature and humidity

Relative humidity tends to be higher in greenhouses than in the open field. This high humidity directly affects floral rewards; the nectar sugar is more dilute since humidity affects rate of evaporative water loss. As a result, nectar sugar concentrations are sometimes below those preferred by honey bees, negatively affecting pollination activity (Dag and Eisikowich, 1999). Furthermore, at high temperatures honey bees lose heat through the evaporative cooling that occurs when they regurgitate nectar from the honey stomach (Heinrich, 1980). Temperature and relative humidity sensors therefore should be placed inside the plant foliage to follow the environmental conditions to which the flower is exposed, and somewhere above the foliage to monitor conditions to which the foragers are exposed.

8.4. Directed air flow

Greenhouses are actively ventilated by forced air ventilation or passively by opening side walls to reduce humidity and overheating that stress plants and promote foliar diseases (Fig. 18). Side walls and screens can also be opened to direct and regulate air flow and velocity within the greenhouse relative to the location of a hive. Air flow direction has been shown to affect honey bee pollination activity and subsequent fruit-set in the greenhouse (Dag and Eisikowitch, 1995), a phenomenon explained by bees' tendency to fly upwind (Friesen, 1973). It is recommended that a wind speed and direction sensor (anemometer) be placed near the hive entrance as well as in the greenhouse in a central location above the plant foliage.

8.5. Limited food resources

The amount of nectar and pollen provided by a crop in an enclosure is generally insufficient for long-term maintenance of honey bee colonies (Free, 1993). Moreover, adverse effects may express in a honey bee colony restricted to a greenhouse monofloral pollen source (Herbert

et al., 1970); pollination activity is curtailed, and colonies may deteriorate in the space of a few weeks and eventually collapse (Kalev *et al.*, 2002). There are different solutions for these nutritional deficits (Dag, 2008). One is to allow the honey bees to forage in the open and in the enclosures on alternating days (Butler and Haigh, 1956). Another is to use double entrance hives with one entrance leading into the enclosure and the other leading outside the enclosure to allow bees to forage and feed on the surrounding flora (Free, 1993). A third possibility is to artificially feed the colony (section 7.2.). This was shown to be efficient in a sweet pepper greenhouse (Kalev *et al.,* 2002).

Fig. 18. Greenhouse ventilation system.

9. Pesticides and pollinators

Negative consequences of pesticide interactions with bees pollinating crops are a serious concern. Methods to assess risk to individual bees and colonies from toxic effects of chemicals are established, and methods are expanding to include sublethal behavioural effects such as disorientation of foragers (see Medrzycki *et al.,* 2013). Obviously any environmental toxins which affect the health of a colony may impact the effectiveness of the colony as a pollinating unit by altering (especially diminishing) foraging activity.

Other effects can come from purposeful use of chemical attractants and repellents on a blooming crop. The effects of such chemicals can be measured using techniques to determine bee densities in whole fields or orchards (sections 5.1. or 5.2.) or, more commonly, in small plots. Similar small-plot techniques can be used to gauge any pollination-related effects from GMO crops that potentially arise from altered secretion of nectar and shedding of pollen.

10. Economic valuation of crop pollination by honey bees

Several methods have been proposed for the economic valuation of crop pollination by honey bees and wild insects. This value has been defined as the cost to replace pollination provided by honey bees or wild insects with other sources (e.g. hand pollination) (Allsopp *et al.*, 2008), the income of crop production attributable to pollination (Morse and Calderone, 2000; Gallai *et al.*, 2009), the net income (income - costs) of crop production attributable to pollination (Olschewski *et al.*, 2006; Veddeler *et al.*, 2008; Winfree *et al.*, 2011), or a consumer surplus approach (Southwick and Southwick, 1992). Considering the studies using these methods, some focused on the effects of the *total* depletion of biotic pollination; however, typical management decisions only produce *partial* changes in biotic pollination (Fisher *et al.*, 2008). Therefore, marginal values are most useful when designing management strategies. Here we describe briefly how to quantify the contribution of adding hives of honey bees to the value of crop production at the local scale, using the net income method (Olschewski *et al.*, 2006; Veddeler *et al.*, 2008; Winfree *et al.*, 2011). The critical variables are the increase in yield realized by the addition of X hives (ΔH).

Valuation methods apply differently at different scales such as global *(Gallai et al.*, 2009), national (Southwick and Southwick, 1992; Morse and Calderone, 2000), regional, subregional, or local. In sections 10.1. and 10.2., we focus on the local scale because this is the level at which management decisions are applied, i.e., the optimal density of hives needed for a certain crop species. At regional or larger scales, lower crop yield produced by massive losses of pollinators can generate compensatory increases in cultivated area to maintain crop production (Garibaldi *et al.*, 2011) or increases in market prices of crops (Winfree *et al.*, 2011), and these are treated in sections 10.3.

10.1. Determining yield in response to specific colony density

The reader is directed to sections 4 and 5 that explain these methods in detail, bearing in mind that for our immediate purpose we are interested in the change in net income given a particular increase in hive number. Considering that increased pollinator abundance should augment yield at a decelerating rate to the point that additional individuals do not further increase (e.g. pollen saturation) (Fig. 19) or even decrease (e.g. pollen excess) yield (Chacoff *et al.*, 2008; Morris *et al.*, 2010; Garibaldi *et al.*, 2011), valuation analysis should include a range of hive densities (i.e. from zero to high numbers). At the very least two situations are needed for comparison: fields with hives *vs.* fields without hives (control). Each treatment should be replicated

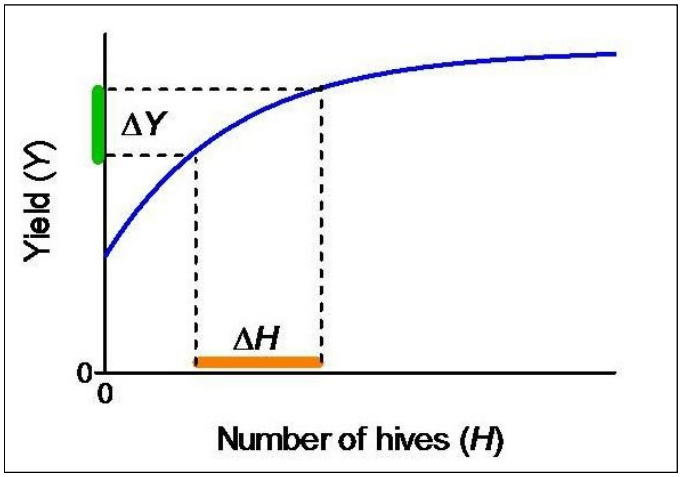

Fig. 19. The marginal benefit to crop yield (e.g. tonnes per ha) of each additional bee colony decreases with colony number. When this marginal benefit equals the cost generated by more yield (variable cost) plus the cost of renting a hive, the net income generated by the addition of the hive is zero and is no longer economically beneficial for the farmer.

with several fields (Prosser, 2010), and the number of necessary fields can be estimated using standard statistical techniques (Anderson *et al.*, 2008). In brief, more fields are necessary if we desire higher statistical power or if we face highly heterogeneous conditions within and between fields. Pollination provided by wild insects should be also measured, as the effect of adding hives of honey bees on crop yield will greatly depend on the "base" level of pollination being provided by wild insects. In addition, the presence of wild insects can enhance honey bees pollination behaviour (Greenleaf and Kremen, 2006; Carvalheiro *et al.*, 2011).

10.2. Response variables and calculations

Net income is the difference between costs from income. We are interested in the change in net income (ΔH) given a particular increase in number of hives (ΔNI):

$$\Delta NI = P * \Delta Y - Cy * \Delta Y - Ch * \Delta H$$

where P is the price that the farmer obtains for each metric tonne of crop, ΔY is the increase in metric tonnes of crop because of the addition of hives (ΔH), Cy is the cost of producing each tonne (i.e. variable costs such as harvest and transportation costs), and Ch is the cost of renting each hive. As mentioned before, at least two treatments are needed to estimate yields without honey bees and subtract them from yields with honey bees ($\Delta Y = Ywith - Ywithout$). In case information for several treatments is available, i.e. several densities of colonies are evaluated, a functional form of yield (Y) with increased number of hives (H) can be estimated. This function should be used to obtain ΔY values for any number of hives within the measured range (Fig. 19).

The data to perform this valuation should be measured at the field scale (e.g. crop yield) or obtained through questionnaires to the farmers and beekeepers (Olschewski *et al.,* 2006). Fruit or seed yield (tonnes per ha) should be measured at ripeness or harvest. The crop price (P) and production costs (including the costs of renting hives) should be obtained from questionnaires.

If honey bees promote yield quality (e.g. bigger and well-formed fruits) in addition to yield quantity (Y), changes in crop prices (***P***) may occur with or without hives. In this case, the price obtained with the desired number of hives should be used in the above formula to estimate *ΔNI* (note that not only ΔY but also all the harvest, ***Y***, can show enhanced quality and price). On the other hand, if losses of honey-bee colonies are replaced with other sources of pollination (e.g. hand pollination), this change in production costs should be also accounted in the above formula. Finally, we must account for some management inputs, such as planting hedge rows with flowering species to improve honey-bee colony health, that incur high initial costs but low costs in subsequent years. Therefore, the temporal scale of analyses, as well as equations used to estimate the net income, will depend on the management practices to be evaluated.

By now it should be evident that the quality of the data gathered has a strong influence on the resulting values for the contribution of honey bees. Several of the ideas discussed here (e.g. replication, scale) can also be applied to other objectives such as the evaluation of the impacts of adding hives on the pollination of surrounding wild vegetation or the diversity and abundance of wild pollinators.

10.3. Economic valuation at larger scales

Crop production (supply) is the product of yield (tonnes ha^{-1}) and cultivated area (ha). Lower crop yield (or slower yield growth over years) generated by the lack of adequate pollination can affect production at regional, national or global scales (Garibaldi *et al.*, 2011). In this case, cultivation of more area to compensate for production losses is a likely outcome and should be included in valuation. Another likely outcome is the increase in the market price for the harvested product (Winfree *et al.*, 2011). Valuation at larger than local scales should also account for the welfare of producers as well as consumers (Gallai *et al.*, 2009; Winfree *et al.*, 2011). In economic terms, the welfare of producers can be described as the producer surplus (here we will focus on the net income approach following Winfree *et al.*, 2011), and the welfare of consumers can be described as the consumer surplus (Fig. 20) (Southwick and Southwick, 1992). In addition, it is important for decision making to consider how the value of honey bee pollination to crops changes spatially across the study region (Chaplin-Kramer *et al.*, 2011; Lautenbach *et al.*, 2012).

Here we discuss quantifying the extent to which honey bee numbers affect crop production value loss or gain at the regional scale. Changes in honey-bee abundance within a region will impact

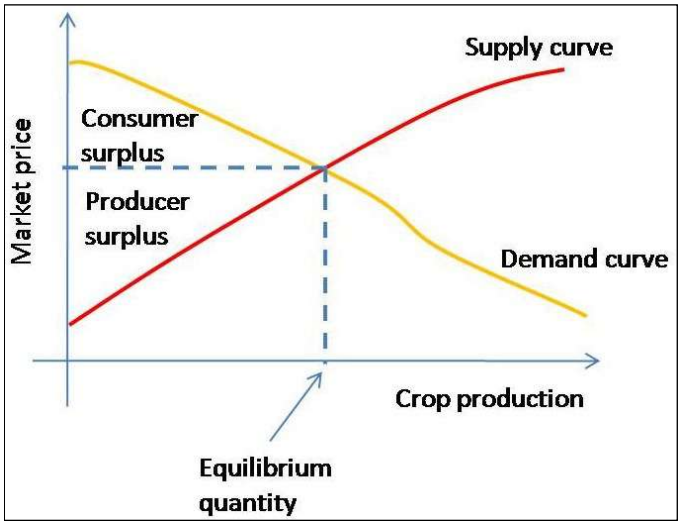

Fig. 20. Consumer surplus occurs because consumers are able to purchase a product at a lower price than the highest price they are willing to pay (presented as area between the demand curve and the market price at the equilibrium quantity). If crop price rises, consumer surplus decreases. Producer surplus occurs because producers sell at a price that is higher than the lowest price they are willing to receive to sell their product (presented as the area between the supply curve and the price at the equilibrium quantity). The functional form of the supply and demand curves are only to exemplify the concept of consumer and producer surplus and need to be estimated from real data for each crop and market.

social welfare (**SW**) in three ways: through the aggregate net income of the crop producers in the affected area (**NIr**), the aggregate net income of producers outside the affected area but sharing the same market (**NIo**), and the consumer surplus (**CS**) (Winfree *et al.*, 2011).

$$SW = NIr + NIo + CS$$

The net income because of a certain variation (suppose losses) in the number of hives (ΔH) for producers within (**NIr**) and outside (**NIo**) an affected area can be estimated similarly to that given above for the local scale:

$$NI = (\Delta P + P) * (\Delta Y + Y) - Cy * (\Delta Y + Y) - (\Delta Ch + Ch) * (\Delta H + H)$$

For producers within the affected area, the loss of honey bees can decrease yield (ΔY) in comparison to the yield previous to the loss of honey bees (Y) and increase crop price (ΔP). Therefore, net income can be reduced because of lower yield on the one hand, but increased because of higher prices on the other. The net outcome will depend on the relative changes in yield and crop prices. In addition, lower yield will reduce the variable costs ($Cy * \Delta Y + Y$) such as costs to harvest and transport crop yield. A significant decrease in the number of honey bee colonies will likely increase the cost of renting each hive ($\Delta Ch + Ch$) and modify the number of hives each producer rents ($\Delta H + H$). For producers outside the affected area, no change in yield ($\Delta Y = 0$) or variable costs ($Cy * \Delta Y = 0$) may happen, but net income is influenced by changes in crop price and probably by changes in the cost of renting a hive. Finally, it is important to note that ΔP will be a function of the amount of crop production (tonnes) that decreases in the affected area in relation to the total production traded at the market and of the ability of producers to increase crop area to compensate for lower production (i.e. the price elasticity of supply) (Fig. 20) (Garibaldi *et al.*, 2011).

Consumer surplus occurs if consumers are willing to pay a price for the crop product that is higher than market price. Therefore, price increases resulting from honey bee losses will reduce consumer surplus (Fig. 20). Estimating the change in consumer surplus requires estimation of the demand curve using questionnaires or historical market data. The estimations presented here for total welfare effect of a certain variation in honey-bee numbers will also require the estimation of ΔP which can be obtained from the price elasticity of supply and the current crop prices (Fig. 20) (for more details see Winfree *et al.*, 2011). The rest of the data required are the same as discussed in sections 10.2. and 10.3.

We have limited ourselves here to a brief introduction of the key factors for analysing the value of honey bees as pollinators of agricultural crops at a regional scale. The social welfare value obtained with these models depends on the quality of the data gathered. Correspondingly, it is important to study how the resulting values for social welfare change with variation in the assumed functional forms or parameter estimates (i.e. sensitivity analyses; the same is true for the local scale valuation presented before). The functional form of the number of hives on crop yield has not been reliably estimated for most crops at field scales; this is a crucial knowledge deficit in our understanding of the benefits of honey bees to agricultural production.

11. Acknowledgements

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