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Do floral resources influence pollination rates and subsequent fruit set in pear (*Pyrus communis* L.) and apple (*Malus* x *domestica* Borkh) cultivars?

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ABSTRACT

Pear and apple are among the main fruit crops worldwide. These species can be planted in mixed orchards, and they both depend on insect pollination for fruit set. As pollinating insects are attracted by the floral resources, we investigated nectar and pollen production and chemical composition in four pear ('Concorde', 'Conférence', 'Doyenné du Comice', 'Triomphe de Vienne') and five apple ('Braeburn', 'Golden Reinders', 'Jonagored', 'Pinova', 'Wellant') cultivars commonly grown in Belgium. We also investigated whether insect flower visitation rate and pollination efficiency are linked to floral resource quantity and quality. The pear cultivars flowered one week before the apple cultivars in early spring, and their flowers were about six times less visited by insects. The visitors foraged more on the pollen of the pear trees and the nectar of the apple trees. Pear flowers produced higher volumes of nectar than apple flowers (1.3–3.2 μl vs. 0.4-0.6 μl), but with lower sugar concentration (9.6%-10.8% vs. 28.3%-36.4%). Pear flowers also produced fewer pollen grains per anther than apple flowers (2425–4937 vs. 3284–7919), but these had higher polypeptide (346–362 μg/mg vs. 216–303 μg/mg), amino-acid (40–77 μg/mg vs. 12–18 μg/mg) and phytosterol (21–47 μg/mg vs. 15–43 μg/mg) concentrations. The foraging behavior of the insects is thus better explained by nectar and pollen quality rather than quantity. Despite the differences in flower visitation rates, pollination of both species resulted in valuable fruit production.

1. Introduction

Apple (*Malus* × *domestica* Borkh.) and pear (*Pyrus communis* L.) are pomaceous fruit-tree species of the Rosaceae family. They are the fourth (76.4 million tons/year) and twelfth (23.6 million tons/year) most productive fruit crops, respectively, worldwide, according to the United Nations Food and Agriculture Organization (FAOSTAT, 2015). They are mainly cultivated in temperate countries, and these species can co-exist in mixed orchards (Díaz et al., 2013). As for most Rosaceous species, they exhibit gametophytic self-incompatibility. Intra-cultivar self-incompatibility makes it necessary to plant inter-compatible cultivars together to ensure fertilization, and seed and fruit set (Delaplane et al., 2000; Tassinari et al., 2003). This impediment represents an obstacle to achievement of high and stable fruit yields (Matsumoto, 2014; Ramírez and Davenport, 2013; Webster, 2002). It is well known that the number and size of seeds affect fruit enlargement and quality in both pear and apple trees, thus increasing the marketability of the fruit (Garratt et al., 2014; Monzón et al., 2004; Sakamoto et al., 2009; Sheffield, 2014). Moreover, as well as the pollinating insects, flowering overlap among compatible cultivars is required to promote effective pollen transfer and facilitate inter-cultivar pollination (Delaplane et al., 2000; Stern et al., 2004). The annual economic value of this insect pollination has been estimated at US $113.4 million and US $208.5 million for the pear and apple industries, respectively (Allsopp et al., 2008).

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Bees are the predominant pollination vectors for both of these species. Therefore, their activity in orchards is essential for fruit production (Delaplane et al., 2000; Gupta, 2005; Ramírez and Davenport, 2013; Stern et al., 2007). Significant positive correlations between bee visitation rates and fruit yield have been reported for both pear and apple trees (Stern et al., 2001, 2004, 2007). Honey-bee hives are often established in orchards to improve the pollination, and the recommended density of bee hives is from one to five hives per ha (Stern et al., 2007). In addition to honey bees, other wild pollinators such as bumble bees, solitary bees, and hoverflies also contribute to pollination for pear and apple trees (Blitzer et al., 2016; Gupta, 2005; Martins et al., 2015; Monzón et al., 2004; Rader et al., 2015; Ramírez and Davenport, 2013; Van den Eijnde, 1996). It has also been shown that pear flowers are considered less attractive to bees than apple flowers (Delaplane et al., 2000; Díaz et al., 2013; Stern et al., 2007), although the basis for this difference in attractiveness is not clearly understood.

Bees visit flowers for the floral resources; namely nectar and pollen. The quality and quantity of these resources greatly affect insect foraging behavior. For bees, nectar and pollen represent unique food sources for both adults and larvae (Michener, 2007). Nectar represents the major energy source, while pollen is one of the prime nutrient resources used for bee larvae (Cnaani et al., 2006; Somme et al., 2014; Vanderplanck et al., 2011, 2014a). Nectar consists largely of sugars (mainly sucrose, glucose, fructose) and water, although it also contains minor constituents, such as amino acids and lipids (Corbet, 2003; González-Teuber and Heil, 2009). Pollen consists mostly of lipids (including phytosterols), polypeptides, and amino acids, and contains vitamins and sugars to a lesser extent (Day et al., 1990; Vanderplanck et al., 2014a,b). Bees cannot synthesize phytosterols de novo, which are the precursors of molting hormones (Behmer and Nes, 2003; Cohen, 2004). Similarly, bees need to assimilate essential amino acids from pollen (de Groot, 1953). Polypeptides have several functional roles in the insect diet, including for binding fats and flavors, for storage, and for immune functions (Vanderplanck et al., 2014a). Defining the quantity and quality of these floral resources is thus of major importance for an understanding of the attractiveness of pear and apple flowers for pollinating insects.

Nectar production and quality have been studied in some pear and apple cultivars, and differences in their attractiveness have partly been attributed to differences in the nectar sugar concentration (Díaz et al., 2013; Farkas and Orosz-Kovács, 2003; Farkas and Zajácz, 2007; Stern et al., 2007; Tóth et al., 2003). Indeed, the sugar content of pear nectar is usually lower (often < 10%–15%) compared to apple (often > 10%) and to other fruit-tree species, such as quince (9.0%–47.5%), cherry (12%–65%), almond (16.0%–32.5%), and peach (often > 50%) (Faoro and Orth, 2011; Farkas et al., 2002a; Farkas and Zajácz, 2007; Jacquemart et al., 2006; Monzón et al., 2004).

However, the chemical compositions of pear and apple pollen have not been analyzed to date, to the best of our knowledge. Thus it is not known to what extent the behavior of pollinating insects can be explained by the chemical compositions of pear and apple pollen. Pollen composition has been reported to define bee foraging strategies in other Rosaceae plant species (Somme et al., 2014). To this end, we investigated the relationships between floral resources, pollination, and fruit production in pear and apple trees. The pollination efficiency was evaluated by recording of the pollinating insect diversity and the flower visitation rates. The quality of the floral resources was estimated based on the chemical content of the nectar (i.e., sugar composition) and the pollen (i.e., polypeptide, amino-acid, phytosterol compositions). Fruit and seed set were subsequently investigated to determine the fruit production.

These parameters were compared for four pear ('Concorde', 'Conférence', 'Doyenné du Comice', 'Triomphe de Vienne') and five apple ('Braeburn', 'Golden Reinders', 'Jonagored', 'Pinova', 'Wellant') cultivars within the same orchard, to standardize the abiotic parameters. These observations allowed us to answer the following questions: (i) To what extent are pollinating insect visitation rates different between pear and apple cultivars? (ii) Do the floral resource quantity and quality differ between these species? (iii) Can the floral resources explain the differences in insect behavior? (iv) Does insect pollination result in valuable fruit production?

2. Material and methods

2.1. Site and plant materials

This study was carried out in 2014, in a 5-ha orchard that belonged to Centre Fruitier Wallon (CEF) in Merdorp (50° 38′ 31″ N; 5° 0′ 15″ E), Belgium. This orchard contained about 2 ha of pear trees planted at 3.75 m × 1.5 m spacing, and 3 ha of apple trees planted at 3.75 m × 1.25 m spacing. The main pear cultivar was 'Conférence', with 'Concorde', 'Doyenné du Comice', and 'Triomphe de Vienne' planted as pollinizers. These pollinizer trees were either in the row at a ratio of 1 pollinizer tree to 16 'Conférence' trees, or they alternated with each 5 rows of 'Conférence' trees. The apple cultivars were 'Braeburn', 'Golden Reinders', 'Jonagored', 'Pinova' and 'Wellant', and they alternated per block of 2–5 rows per cultivar. All of the cultivars were at least semi-compatible (i.e., each cultivar differed by at least one S-allele from the other cultivars; Broothaerts et al., 2004; Goldway et al., 2009; Matsumoto, 2014; Quinet et al., 2014) and showed overlapping flowering (Fig. 1). All of the orchard management practices were identical and similar to those for commercial production. During the flowering period, five honey-bee (*Apis mellifera*) hives of about 40,000 individuals per hive and two bumble-bee (*Bombus terrestris*) hives of 3 colonies each, at about 150 individuals per colony (multihive, Biobest, Westerlo, Belgium), were established in the orchard at the beginning of the pear flowering period (April).

2.2. Flowering phenology, fruit development, and fruit production

For each cultivar, 20 inflorescences on 2-year-old wood branches were selected and followed from flowering until harvest. The numbers of flowers at anthesis were recorded each day or every other day through the entire flowering period. The numbers of fruitlets and fruits at harvest were recorded, in May and September, respectively. The fruit growth was followed once a month. At harvest, fruit size, fruit weight, and number of viable and aborted seeds per fruit were quantified on 30 fruits per cultivar. Seed viability was confirmed using the tetrazolium test (Kearns and Inouye, 1993).

2.3. Insect observations and pollination efficiency

Flower visitors were recorded on sunny days during the whole flowering period (minimum 4 days per cultivar) between 10:00 h and 16:00 h. For each species, the observations were performed for the different cultivars on the same days under the same weather conditions. The number of open flowers, the number and identity of insects visiting the flowers (classified as honey bees, bumble bees, solitary bees, hoverflies, other minor visitors), the number of flowers visited per insect, and the time spent per flower were recorded per 'plot' over periods of 10 min. A plot consisted of 5–10 branches of a tree. The observations were made over a total of 150 min to 200 min per cultivar for the pear trees, and 100 min per cultivar for the apple trees. A total of 175 and 305 insects were observed for pear flowers and apple flowers, respectively. The flower visitation rates were calculated as the ratios between the number of flowers visited by insects in a plot per hour and the total number of open flowers in the plot.

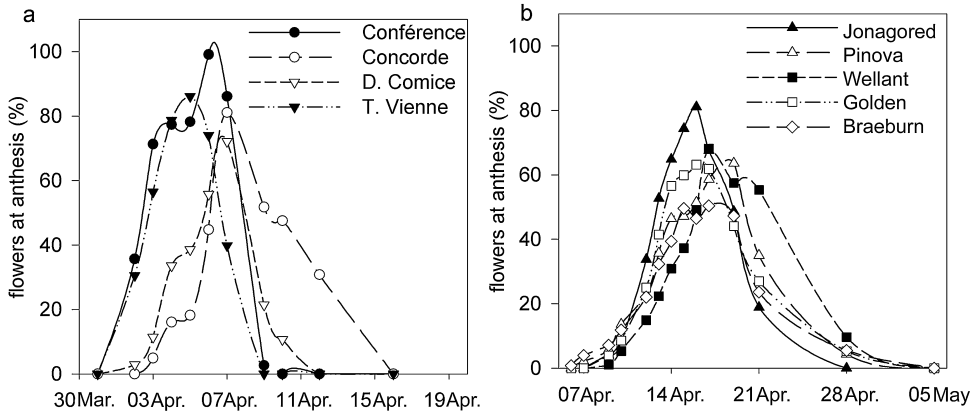

Fig. 1. Cumulative percentage of flowers at anthesis in the pear (a) and apple (b) cultivars.

To determine the honey-bee and bumble-bee fidelity, we sampled the pollen loads from 10 honey bees and 10 bumble bees returning to the hive during the full flowering of the pear and apple trees. After the bees had been immobilized in a bee marking cage, one of the two pollen loads was carefully removed using a toothpick. In the laboratory, these pollen loads were acetolyzed (Erdtman, 1960; modified) and the pollen grains were identified under light microscopy (Eclipse E400; Nikon, Amsterdam, The Netherlands). The pollen identification was based on a reference collection from the Université catholique de Louvain, an identification key (Reille, 1992), and a comprehensive list of the flowering plants in the vicinity of the orchard. At least 400 randomly chosen pollen grains per pollen load were identified and counted.

To determine pollination efficiency, 20 flowers per cultivar were randomly harvested at the end of the flowering period. Pollen grains and pollen tubes were examined under fluorescence microscopy with 420 nm to 440 nm excitation filter and 480 nm emission filter (Eclipse E400; Nikon, Amsterdam, The Netherlands), according to Jacquemart et al. (2006). The five styles were dissected from each flower and placed in 1.0 M NaOH for 4 h, to soften them. The styles were then rinsed in distilled water, and crushed in 0.1% (v/v) aniline blue solution in 1.0 M KH_2PO_4, pH 9.0. The number of pollen tubes in the styles of each flower was estimated and classified into one of three abundance categories, as: 1–10 pollen tubes, 10–20 pollen tubes, or >20 pollen tubes.

2.4. Nectar sampling and chemical analyses

To allow comparisons under similar conditions, the nectar sampling was carried out on the same days for the different cultivars of the same species. The sampling was performed on freshly opened flowers (10–15 flowers per cultivar, per day) on two different days (sunny; temperature, 17–20 °C during sampling) for each species. The flowers were covered with exclusion bags (of cotton mesh) for 24 h before sampling to prevent visits by insects. The nectar was collected using glass capillary tubes of 0.5 μl, 1 μl or 5 μl volumes, depending on the quantity of the nectar (Hirschmann® Laborgerate, Eberstadt, Germany). The nectar volumes were estimated by measuring the length of the nectar column in the capillary tube. The nectar sugar concentrations were measured using a low-volume hand refractometer (Eclipse Handheld Refractometer; Bellingham & Stanley Ltd, Tunbridge Wells, UK), and are expressed as percentages of sucrose per nectar mass (w/w). To determine the total sugar content per flower of the nectar, the sugar concentrations in percent were converted to the sugar concentrations in mg/μl according to the formula: $y = 0.00226 + (0.00937 \times) + (0.0000585 \times^2)$, where y is the sugar concentration in mg/μl, and x is the sugar concentration in percent (Dafni et al., 2005). The total sugar content of the nectar per flower (μg) was then calculated as the volume of nectar (μl) multiplied by the sugar concentration (mg/μl) × 1000. The sugar composition of the nectar was determined using high performance liquid chromatography (Shimadzu HPLC system) coupled to a refractometer (RID10A; Shimadzu, 's-Hertogenbosch, The Netherlands) using a gold amino column (150 × 4.6 mm; Hypersil; Thermo Scientific, Aalst, Belgium) at 26 °C. The mobile phase was 83% (v/v) acetonitrile in water, and the flow rate was 1.0 ml/min. The nectar composition analyses were performed in the Groupe de Recherche en Physiologie Végétale (Université catholique de Louvain, Louvain-la-Neuve, Belgium).

2.5. Pollen sampling and chemical analyses

For pollen-grain quantification, the anthers were collected from 10 flowers per cultivar at full balloon stage. The anthers were individually crushed in a microcentrifuge tube (Eppendorf, Germany) that contained 200 μl Alexander's stain (Alexander, 1969). These were then mixed and sonicated to disperse the pollen grains in the solution. The number of pollen grains per anther in 10 μl was counted in triplicate under light microscopy (Eclipse E400; Nikon, Amsterdam, The Netherlands).

For the chemical analyses, the stamens were collected from hundreds of freshly opened flowers for each cultivar, and dried at room temperature for 12 h. The pollen was then removed using a sieve (Sieve 3″, Brass-Stainless, Full Height, 80 μm). The pure pollen samples were lyophilized before the analysis of the polypeptide, amino-acid, and phytosterol concentrations and compositions. Each analysis was performed in triplicate per cultivar.

The polypeptide concentrations (molecular weight >10,000 Da) were quantified from 5 mg dry pollen, following the method described by Vanderplanck et al. (2014a). The polypeptide purification protocol combined washes and a phenol/sodium dodecyl sulfate extraction. Quantification of the total protein was performed using BCA Protein Assay kits (Pierce, Thermo Scientific), according to manufacturer instructions.

The amino-acid concentrations were quantified from 3 mg dry pollen, following the method described by Vanderplanck et al. (2014b). Total amino acids and free amino acids were extracted separately and measured using ion-exchange chromatography (Biochrom 20 plus amino-acid analyser) at the University of Liège (Gembloux, Belgium). For both extractions, norleucine was used as the internal standard, allowing for further amino-acid quantification. The relative levels of the individual amino acids are expressed as percentages of the total amino-acid content.

The phytosterol concentrations were quantified from 15 mg dry pollen, following the method described by Vanderplanck et al. (2011). After extraction and derivatization of the sterols into their

respective trimethylsilyl ethers, these were separated by gas-liquid chromatography at the University of Liège (Gembloux, Belgium). The total phytosterol content was determined considering all of the peaks of sterols eluted between cholesterol and betulin. The relative levels of the individual sterols are expressed as percentages of the total sterol content.

2.6. Statistical analyses

All of the analyses were conducted in R version 3.0.2 (RDevelopment, 2012). The flower visitation rates, pollen and nectar production and compositions (i.e., polypeptides, amino acids, sterols, sugars) and fruit (weight, size, number of seeds) parameters were compared among the species and cultivars using one-way analyses of variance (one-way ANOVA). The normality of the data was estimated using Shapiro–Wilk tests, and homoscedasticity was verified using Levene's tests. The data were transformed when required, to ensure normal distributions, and Welch's correction was applied when homoscedasticity was not met. The analyses of the fruit parameters were weighted by the number of fruits per tree at harvest. Post-hoc analyses were performed using Tukey's tests to investigate the differences among the cultivars within a species. To determine the differences between the pollen compositions (i.e., amino-acid and sterol profiles), we conducted per MANOVA (Euclidean dissimilarity index, 999 permutations, 'adonis' command) and multiple pair-wise comparisons with Bonferroni's adjustment after testing for multivariate homogeneity ('betadisper' command) ('vegan' package; Oksanen et al., 2007). Differences were visually assessed through UPGMA clusters using Euclidean dissimilarity indices, and heatmaps were constructed ('heatmap.2' command, 'gplots' package). We assessed the uncertainty in hierarchical cluster analysis with p-values calculated via multiscale bootstrap resampling ('pvclust' package; Suzuki and Shimodaira, 2006). We performed principal component analysis (PCA) using the 'FactoMineR' package, to compare the pollen and nectar compositions of the pear and apple cultivars, as well as the fruit parameters. Visitation rates were added as a supplemental variable in the PCA, to illustrate the relationships with the floral resources and fruit parameters. Proportions of visiting insects, conspecific pollens in pollen loads, pollen tubes in the styles, and fruit set were compared among species and cultivars using chi-square tests. If not indicated otherwise, the data are presented as means ± standard errors.

3. Results

3.1. Insect visitation rates are lower for pear than apple trees

The pear cultivars flowered about 1 week before the apple trees, regardless of cultivar, and the complete flowering period in pear was half the duration of the apple cultivars (Fig. 1; $F_{1,7} = 88.11$, $p < 0.0001$). As a result, overlap between pear cultivar flowering (duration, 4–5 days) was lower than for apple (duration, 18 days) (Fig. 1).

During the flowering periods, honey bees and bumble bees that mainly originated from the hives in the orchard were the main visitors for both tree species (Fig. 2a). They accounted for 61.9% ± 4.9% and 15.8% ± 3.1%, respectively, of the total insect visitors. The other observed pollinating insects were solitary bees (4.0% ± 1.4%) and hoverflies (9.7% ± 1.1%). The proportion of honeybees was lower for the pear flowers compared to the apple flowers (50.3% ± 7.0% vs. 71.2% ± 4.5%; $\chi^2 = 31.7$, $p < 0.0001$), while the proportion of hoverflies was higher for the pear flowers than the apple flowers (15.6% ± 6.0% vs. 5.0% ± 1.0%; $\chi^2 = 82.6$, 8, $p < 0.0001$). The proportion of the different groups of insects also varied among the

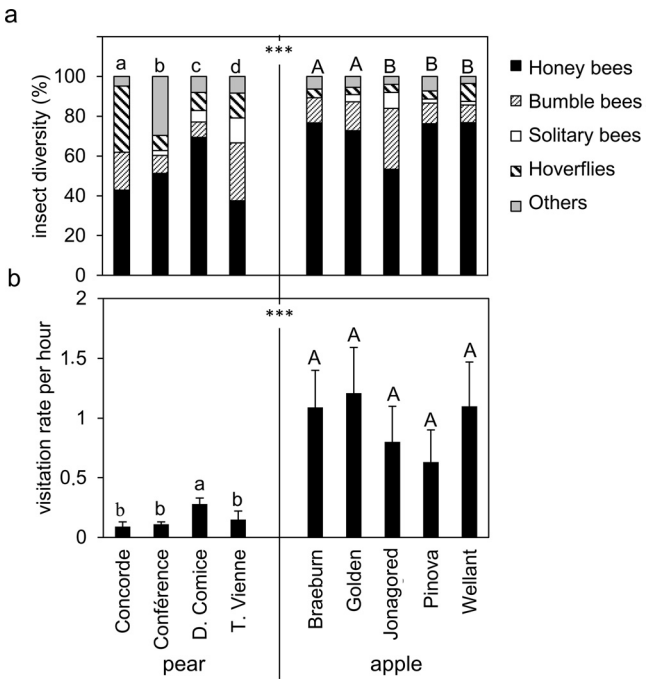

Fig. 2. Insect visitation of the nine studied pear and apple cultivars. (a) Diversity of insects visiting the flowers. (b) Flower visitation rate per hour. Significant differences between pear and apple are indicated by *** ($p < 0.0001$). For each species, cultivars followed by different letters are significantly different at $p < 0.05$.

cultivars within a same species (Fig. 2a; $\chi^2 = 100.1$, 12, $p < 0.0001$ for pear and $\chi^2 = 43.16$, 16, $p = 0.0003$ for apple).

Under these orchard conditions, the pear flowers were about six times less visited by insects than the apple flowers ($F_{1,28.6} = 42.19$, $p < 0.0001$; Fig. 2b). The flower lifetimes were from 4.1 days to 5.4 days in pear, and 5.4 days to 7.9 days for the apple cultivars. Therefore, on average, and under ideal pollinating conditions (assuming 7 h of insect activity per day), during their lifespan, the pear flowers were visited 5 times, and the apple flowers, 45 times. For the pear trees, flowers of 'Doyenné du Comice' were more visited compared to the other cultivars ($F_{9,109} = 7.94$, $p < 0.0001$; Fig. 2b). No differences in the visitation rates were observed among the apple cultivars ($F_{4,19} = 0.37$, $p = 0.8254$; Fig. 2b).

Honey bees and bumble bees showed high fidelity to the pear and apple flowers despite the presence of herbaceous ruderals in the vicinity of the orchard. The pollen loads from honey bees and bumble bees contained 98.1% and 75.0% pear pollen, respectively, and 66.6% and 64.3% apple pollen, respectively. However, the pollen composition in pollen loads varied depending on the insect ($\chi^2 = 3884.8$, df = 39, $p < 0.0001$). The honey bees collected also some pollen from *Taraxacum* spp. (12%) during the pear-tree flowering period, and from *Salix* (3%) and Brassicaceae species (30%) during the apple-tree flowering period. The bumble bee pollen loads also contained pollen of *Salix* spp. (25%, 6%; during pear and apple tree flowering, respectively) and Brassicaceae species (11%, during apple tree flowering).

3.2. Floral resources differ between pear and apple trees

With the aim to investigate whether the floral resources can explain the differences in visitation rates of the pear and apple flowers, we analyzed the nectar and pollen production and composition. PCA showed that the pear and apple trees clustered separately (Fig. 3b). Axis 1 represented 51.62% of the variance (Fig. 3a). This was mainly explained by the amino-acid composition, and it highlighted the differences between the cultivars within each of the

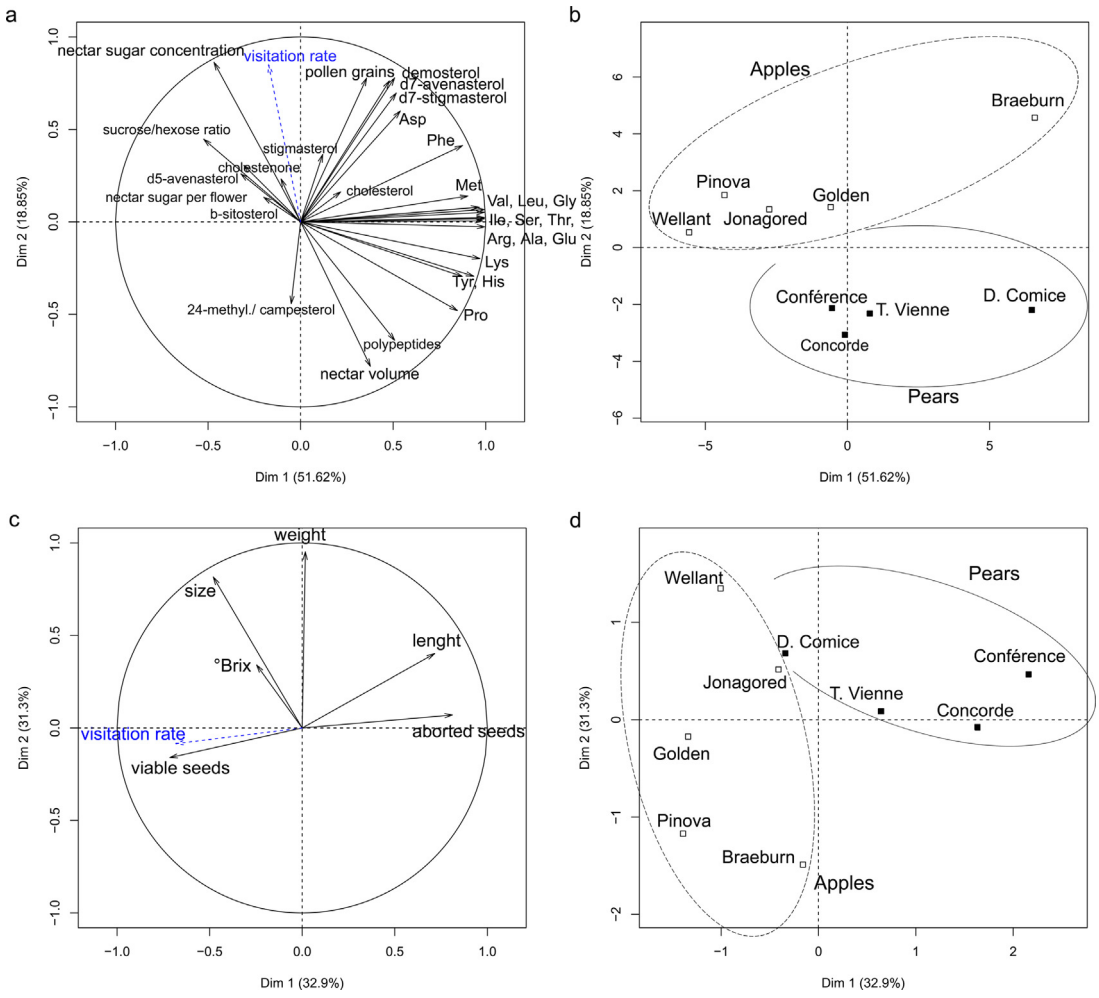

Fig. 3. Principal component analysis of the composition of the floral resources (a, b) and of the fruit parameters at harvest (c, d) for the nine studied pear and apple cultivars. (a, c) Variable graphs: the visitation rates per flower added as supplemental variable and not used for the calculation of the PCA. (b, d) Individual graphs showing the mean values per cultivar; cultivars belonging to the same species are surrounded.

species. Axis 2 accounted for 18.85% of the variance, and this separated the pear and apple trees based on the nectar and pollen compositions (Fig. 3a, b).

3.2.1. Nectar

The pear cultivars produced 3–4 times more nectar per flower than the apple cultivars (2.3 ± 0.3 µl vs. 0.6 ± 0.1 µl; $F_{1,177.6} = 44.39$, $p < 0.0001$; Fig. 4a), while the pear nectar had three times lower sugar concentration ($11.7\% \pm 0.6\%$ vs. $33.2\% \pm 0.8\%$; $F_{1,231.2} = 473.04$, $p < 0.0001$; Fig. 4b). Differences among cultivars were nevertheless observed for both nectar volume and sugar concentration within each species (Fig. 4a, b; $F_{3,111} = 4.59$, $p = 0.0046$, $F_{3.43,4} = 3.26$, $p = 0.0303$ for pear and $F_{3,131} = 3.55$, $p = 0.0088$, $F_{4,60.22} = 9.73$, $p < 0.0001$ for apple, respectively). Overall, the total sugar content in the flower nectar was lower in pear (178 ± 16 µg) than apple (228 ± 21 µg) ($F_{1,249} = 4.16$, $p = 0.0424$; Fig. 4c). This difference was mainly due to the high sugar content of the nectar of the flowers of the apple 'Pinova' cultivar, as the other apple cultivars did not significantly differ from the pear cultivars (Fig. 4c). However, differences in nectar sugar content were observed among cultivars within each species ($F_{3,111} = 4.23$, $p = 0.0072$ for pear and $F_{4,61.11} = 4.18$, $p = 0.0047$ for apple). We observed sucrose, glucose and fructose in the nectar of the pear and apple cultivars (Fig. 4d). Hexoses were dominant in nectar of both species and the pear nectar contained less sucrose than the apple nectar ($F_{1,27.6} = 12.73$, $p = 0.0013$; Fig. 4d). However, no differences were observed among cultivars for a same species ($F_{3,12} = 2.02$, $p = 0.1656$ for pear and $F_{4,23} = 2.80$, $p = 0.0595$ for apple).

3.2.2. Pollen

Overall, pear cultivars produced half as many pollen grains per anther than the apple cultivars ($F_{1,175.2} = 87.23$, $p < 0.0001$; Fig. 4e). Among the cultivars, 'Doyenné du Comice' produced more pollen grains per anther than the other pear cultivars ($F_{3,25.5} = 27.89$, $p < 0.0001$; Fig. 4e), while for the apple cultivars, the highest number of pollen grains were observed for 'Braeburn', 'Golden' and 'Jonagored' ($F_{4,56.9} = 43.57$, $p < 0.0001$; Fig. 4e).

For the pollen quality, in terms of the polypeptides, the pear pollen showed higher concentrations than the apple pollen (53.5 ± 3.1 vs. 18.91 ± 0.94 µg/mg; $F_{1,120} = 132.13$, $p < 0.0001$; Fig. 4f), as also for the total amino-acid concentration (354 ± 4 vs. 223 ± 11 µg/mg; $F_{1,16.85} = 119.61$, $p < 0.0001$; Fig. 4g) and essential amino-acid relative proportion ($44.2\% \pm 0.4\%$ vs. $38.8\% \pm 0.9\%$, $F_{1,16.62} = 28.18$, $p < 0.0001$; Supplemental Table A.1, A.2). The polypeptide concentration varied among cultivars in both pear and apple pollen ($F_{3,28.18} = 7.40$, $p = 0.0008$ for pear and $F_{4,28.4} = 13.43$, $p < 0.0001$ for apple; Fig. 4f) while amino-acid concentration only varied among cultivars in apple ($F_{3,8} = 0.74$, $p = 0.5568$ for pear and $F_{4,9} = 8.96$, $p = 0.0033$ for apple; Fig. 4g). The major amino acids were asparagine and glutamine in both the pear and apple pollen

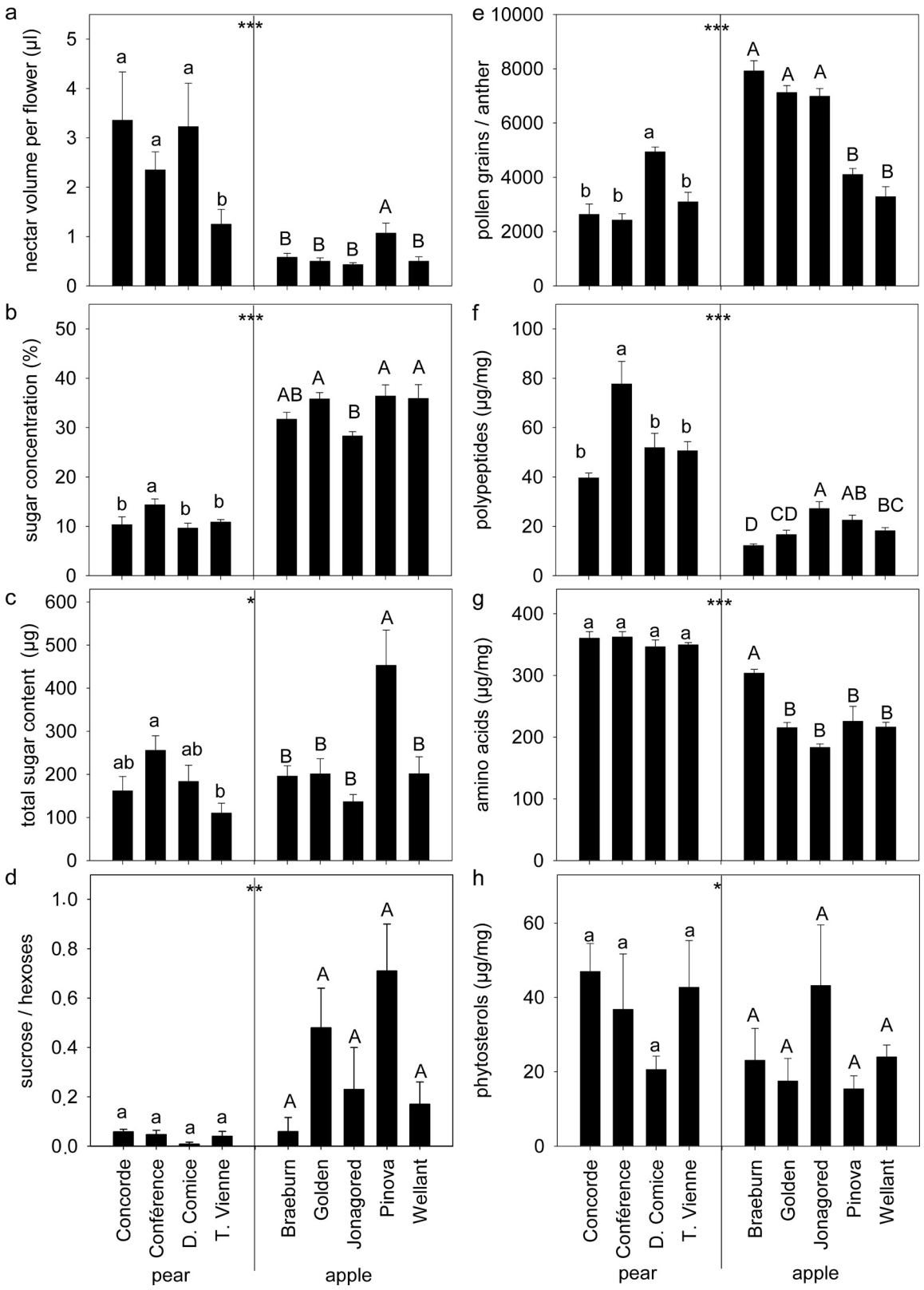

Fig. 4. Floral resources of the nine studied pear and apple cultivars. (a) Nectar volume per flower. (b) Sugar concentration in nectar. (c) Total sugar content in nectar per flower. (d) Sucrose/hexose ratio of nectar. (e) Number of pollen grains per anther. (f) Total polypeptide, (g) amino-acid, and (h) phytosterol concentrations in pollen. Significant differences between pear and apple are indicated by * ($0.05 < p < 0.01$), ** ($0.01 < p < 0.001$), *** ($p < 0.0001$). For each species, cultivars followed by different letters are significantly different at $p < 0.05$.

(Supplemental Table A.1, A.2). The relative levels of asparagine were lower in pear than apple pollen (13.5% ± 0.3% vs. 25.1% ± 1.4%; $F_{1,14.38} = 62.77$, $p < 0.0001$). The total phytosterol concentration was higher in pear pollen than in apple pollen (36.7 ± 5.4 vs. 23.8 ± 4.2 µg/mg; $F_{1,82} = 4.42$, $p = 0.0472$; Fig. 4h) and its concentration was stable whatever the cultivars within a same species

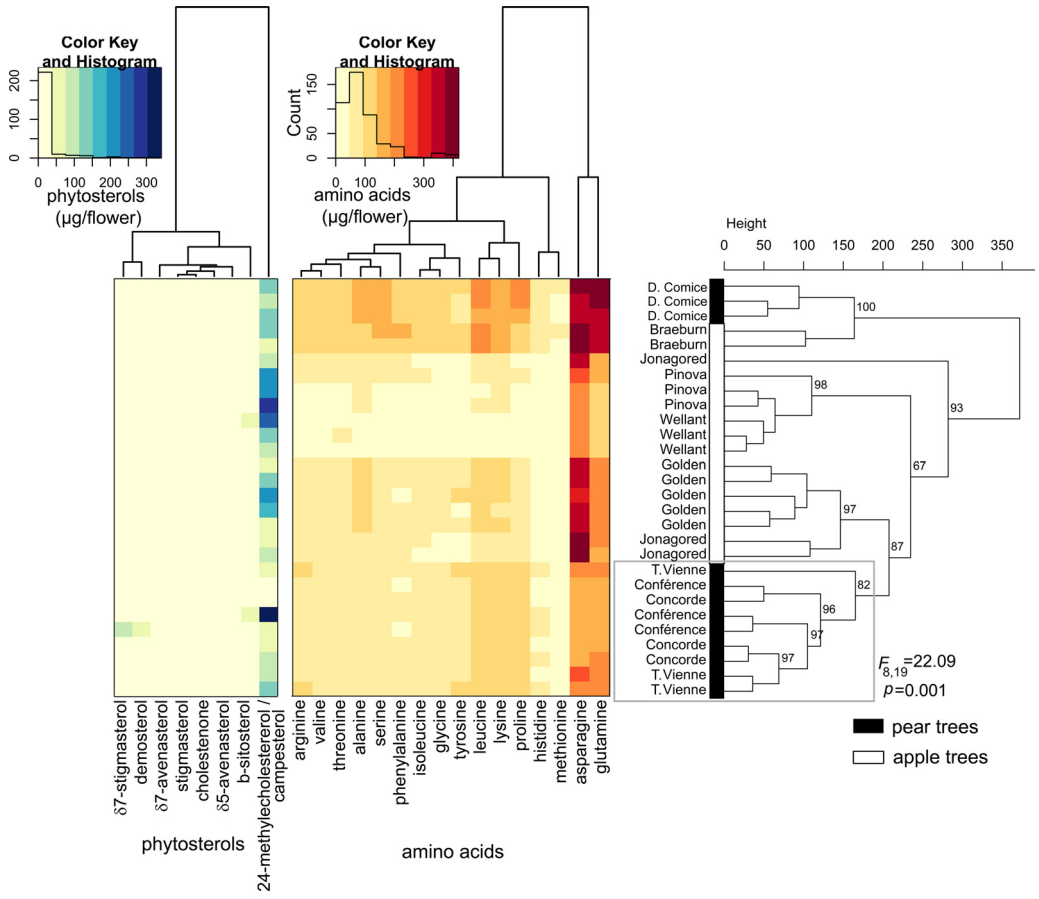

Fig. 5. Pollen compositions of pear and apple cultivars per flower. UPGMA clustering using Euclidean dissimilarity index based on pollen amino-acid and sterolic compositions per flower, and the corresponding heat maps. The values near nodes indicate the multiscale bootstrap resampling. The grouping according to PerMANOVA tests is surrounded in grey on the clustering.

($F_{3,8}$ = 1.18, p = 0.3758 for pear and $F_{4,7}$ = 1.44, p = 0.3141 for apple; Fig. 4h). Methylenecholesterol/campesterol were the main phytosterols in both the pear and apple pollen, although these were at higher relative levels in the pear pollen (85.8% ± 3.3% vs. 61.1% ± 7.3%, $F_{1,12.81}$ = 7.12, p = 0.0195; Supplemental Table A.3, A.4). On the other hand, the pear pollen contained lower relative levels of δ7-stigmasterol (0.9% ± 0.6% vs. 10.3% ± 3.9%, $F_{1,13.50}$ = 5.19; p = 0.0395) and δ7-avenasterol (0.1% ± 0.05% vs. 3.0% ± 1.0%, $F_{1,11.22}$ = 10.21, p = 0.0083; Supplemental Table A.3, A.4) than the apple pollen.

The insect behavior might be more affected by the absolute quantity of pollen compounds available per flower, rather than by their relative concentrations. We thus compared the pollen resources (i.e., amino-acid and sterol composition) produced per flower for all of the cultivars ($F_{8,19}$ = 22.09, p = 0.001; Fig. 5). Except for 'Doyenné du Comice', the pear cultivars were clustered together (Fig. 5; 'Conférence' vs. 'Concorde', $F_{1,4}$ = 1.89, p = 0.303; 'Conférence' vs. 'Triomphe de Vienne', $F_{1,4}$ = 3.23, p = 0.173; 'Concorde' vs. 'Triomphe de Vienne', $F_{1,4}$ = 7.10, p = 0.051), whereas 'Doyenné du Comice' was closer to the apple cultivar 'Braeburn' ($F_{1,3}$ = 6.69, p = 0.061; Fig. 5). The other apple cultivars were equally similar to one another as to the pear cultivars, according to the pair-wise perMANOVA comparisons. The clustering was mainly explained by the amino-acid compositions. The pollen amino-acid compositions varied both among the cultivar pollens ($F_{8,19}$ = 77.35, p = 0.001; Fig. 5) and between the pear and apple pollens ($F_{1,26}$ = 6.38, p = 0.009). The pollens of the pear 'Doyenné du Comice' and the apple 'Braeburn' had the highest amino-acid content per flower (2.4–2.5 mg total amino acids), which was lowest for the apple 'Pinova' and 'Wellant' pollens (0.7-0.9 mg total amino acids); the pollen amino-acid content per flower of the other cultivars ranged from 1.2 mg to 1.6 mg. In general, the cultivars also differed according to their pollen phytosterol compositions ($F_{8,19}$ = 3.99, p = 0.003; Fig. 5), although no differences were observed between the pear and apple trees ($F_{1,26}$ = 1.42, p = 0.231). This showed that the differences were greater within each species (i.e., pear or apple) rather than between the species (Fig. 5). The total sterol content ranged from 125 µg to 193 µg per flower for the pear pollen, and from 71 µg to 304 µg per flower for the apple pollen. Thus, in summary, the compositions of the pollen per flower varied among the cultivars, and differences between the species was supported by the amino-acid content, and not by the phytosterol content.

3.3. Fruit production in pear and apple trees

To investigate whether the flower visitation rates resulted in valuable fruit production, we analyzed the subsequent fruit development. We first investigated the pollen grain deposits on the stigmas and the pollen-tube growth in the styles. All of the observed flowers contained five carpels and a total of 10 ovules. Most of the analyzed styles contained more than 20 growing pollen tubes (χ^2 = 55.8, df = 12, p < 0.0001; Fig. 6a), which suggested that, in general, the pollination was efficient. For the pear cultivars, pollen tube growth was lower in 'Triomphe de Vienne' as 25% of the styles did not contain pollen tubes (χ^2 = 13.0, df = 6, p = 0.0425; Fig. 6a). Although some signs of self-incompatibility were observed, insect pollination resulted in more than 75% fruitlet set in the pear and apple cultivars, with the exception of 'Triomphe de Vienne'

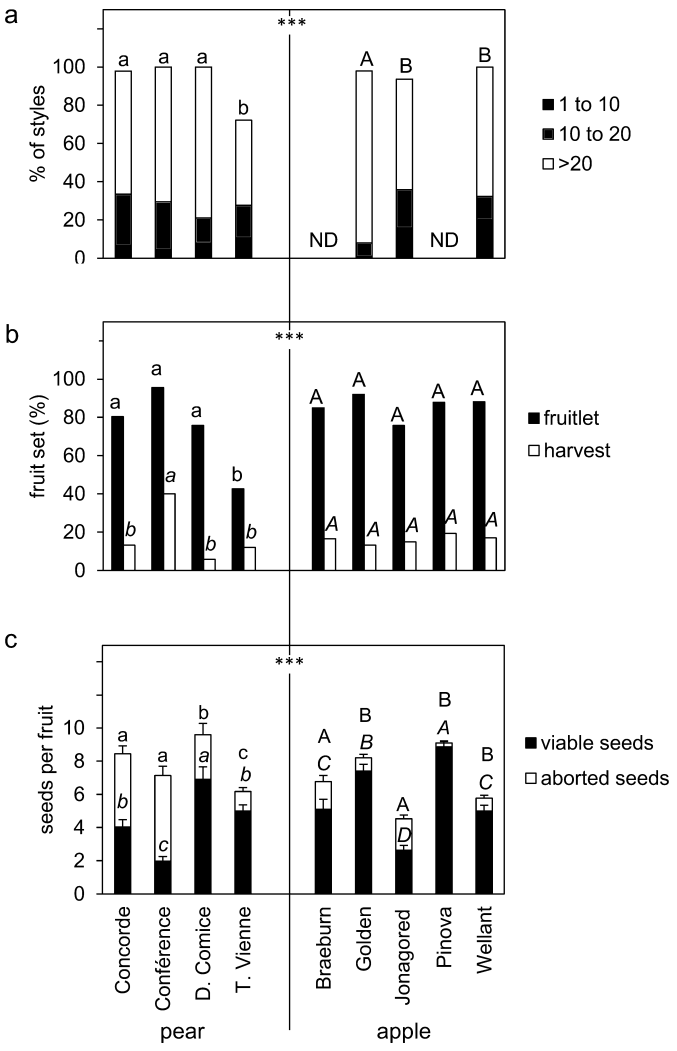

Fig. 6. Pollen tube growth, fruit and seed set of the nine studied pear and apple cultivars. (a) Percentage of styles with growing pollen tubes, according to numbers of pollen tubes per style. (b) Fruit set, calculated as the percentage of flowers that initiated fruit development 2 weeks after pollination (fruitlet) that resulted in a fruit at harvest (harvest). (c) Number of viable and aborted seeds per fruit at harvest. Significant differences between pear and apple are indicated by * ($0.05 < p < 0.01$), ** ($0.01 < p < 0.001$), *** ($p < 0.0001$). For each species, cultivars followed by different letters are significantly different at $p < 0.05$.

(43%) ($\chi^2 = 24.6$, df = 8, $p = 0.002$; Fig. 6b). The fruit set at harvest ranged between 6% and 40% for the pear cultivars ($\chi^2 = 39.1$, df = 3, $p < 0.0001$; Fig. 6b), and between 13% and 19% for the apple cultivars ($\chi^2 = 1.77$, df = 4, $p = 0.777$; Fig. 6b).

Insect pollination also affected seed set which varied among species ($F_{1,259} = 80.01$, $p < 0.0001$; Fig. 6c) and cultivars ($F_{3,107} = 18.92$, $p < 0.0001$ for pear and $F_{4,144} = 29.09$, $p < 0.0001$ for apple; Fig. 6c). For pear, the mean number of viable seeds per fruit ranged from 2.0 ± 0.3 for 'Conférence' to 6.9 ± 0.8 for 'Doyenné du Comice' (Fig. 6c). For apple, the mean number of viable seeds per fruit ranged from 2.6 ± 0.3 for 'Jonagored' to 8.9 ± 0.4 for 'Pinova' (Fig. 6c). The proportion of aborted seeds was higher for pears than for apples (3.4 ± 0.3 vs. 1.1 ± 0.1; $F_{1,259} = 148.47$, $p < 0.0001$), and it was particularly high in the pears 'Concorde' and 'Conférence' (Fig. 6c). In any event, the number of viable seeds correlated with the flower visitation rate, as indicated by the PCA analysis of the fruits (Fig. 3c, d).

Based on the number of fruits per tree and the fruit weight (Supplemental Table A.5), the total production per tree for pear cultivars ranged between 15 kg ('Concorde') and 36 kg ('Triomphe de Vienne'), and for apple, between 19 kg ('Golden') and 30 kg ('Braeburn').

4. Discussion

4.1. The floral resources of pear and apple trees explain the flower visitation rates

Insect diversity and insect fidelity to pear and apple flowers were observed in the orchard suggesting that insect pollination could be efficient in our growing conditions. Honey bees are often considered as the main pear and apple pollinators, although solitary bees and bumble bees also contribute to pollination (Blitzer et al., 2016; Gupta, 2005; Martins et al., 2015; Monzón et al., 2004; Ramírez and Davenport, 2013; Van den Eijnde, 1996). Moreover, increase in pollinating insect diversity was shown to provide better pollination services in fruit crops (Blitzer et al., 2016; Gupta, 2005; Martins et al., 2015). High fidelity to pear and apple flowers has been previously reported in some studies (Benedek et al., 1998; Webster, 2002), while other studies have reported that insects can be more attracted by the ruderal plants in the orchard, such as *Taraxacum* and *Trifolium*, instead of by pear pollen (Faoro and Orth, 2015; Free, 1993).

Despite the higher fidelity of honey bees and bumble bees to pear flowers, they were less visited than apple flowers in our orchard. It has been previously reported that pear flowers are less attractive for bees than apple flowers (Farkas and Orosz-Kovács, 2003; Stern et al., 2007). This difference in attractiveness could be partly explained by the pear and apple floral resources. Correlation between bee visitation rates and sugar concentrations of nectar have been reported for both pear and apple trees (Benedek and Nyeki, 1997; Farkas et al., 2002b). Our observations confirmed that the sugar concentration of pear nectar was lower compared to apple as previously stated for other pear (often <10%–15%) and apple (often >10%) tree cultivars (Faoro and Orth, 2011; Farkas et al., 2002a; Farkas and Zajácz, 2007; Jacquemart et al., 2006; Monzón et al., 2004; Sharifani and Jackson, 2004). Bees do not taste the nectar as sweet if the level of sucrose is <4%, or if the mixture of glucose and fructose is more dilute than 8% to 9% (Tóth et al., 2003), which explains the low attractiveness to the bees of the pear nectar. The relative proportion of the different sugars in nectar also affects the flower attractiveness. With the exception of 'Pinova', all of the present cultivars produced hexose-dominant or hexose-rich nectar. These data show some contrast to previous observations. Some pear cultivars have indeed been reported to contain only glucose and fructose in their nectar while nectar of most of the previously studied apple cultivars belonged to the sucrose-rich group (Farkas and Orosz-Kovács, 2003; Orosz-Kovács et al., 1997; Tóth et al., 2003). Nectar that is sucrose-rich is highly attractive to honey bees and bumble bees (Percival, 1961).

However, the nectar composition alone is not sufficient to explain the observed visitation rates and insect behavior also depends on pollen quantity and quality. If pear cultivars produced less pollen grains per flower than apple cultivars, they produced better quality pollen (i.e., higher polypeptide, amino-acid and sterol concentrations). Polypeptide, amino-acid and sterol concentrations of pear and apple pollen are in the range of those obtained for other Rosaceae using the same techniques (Somme et al., 2014; Vanderplanck et al., 2014b). For the pear pollen, these concentrations are also similar to pollen from *Cytisus scoparius* (Fabaceae) and *Sorbus aucuparia* (Rosaceae), which are considered as good quality pollen for the development of bumble-bee colonies (Vanderplanck et al., 2014b). The pollen amino-acid composition has been reported to be a useful indicator of diet performance in bees (Moerman et al., 2015). For both species, the distributions of the different essen-

tial amino acids were similar to the ideal composition of essential pollen amino acids for bees ($\chi^2 = 5.7$, 16, $p = 0.99$; Supplemental Table A.3, A.4; de Groot, 1953; Weiner et al., 2010). Moreover, asparagine and glutamine, which were the dominant amino acids in the pollen of both species, are important as energy and nitrogen sources for bees (Chapman, 2012; Roulston and Cane, 2000). Pear and apple pollen also could be sources of phytosterols for bees. In particular, 24-methylenecholesterol is an essential sterol in bee metabolism, and it favors molting and the development of ovaries (Human et al., 2007; Vanderplanck et al., 2014a). 24-methylenecholesterol/campesterol were the main phytosterols in pollen of both species and they were especially abundant in pears. However, the apple cultivars 'Braeburn' and 'Pinova' were rich in δ7-sterols which have been reported to be detrimental to herbivorous insects that lack the enzymes to convert them to δ5-sterols (Janson et al., 2009; Sedivy et al., 2011).

Overall, the pear cultivars produced better quality pollen (i.e., higher polypeptide, amino-acid and sterol concentrations), and the apple cultivars produced better quality nectar (i.e., higher total sugar and sucrose concentrations), which is consistent with the observation that the pollinating insects mainly visited pear flowers for pollen and apple flowers for nectar (Calzoni and Speranza, 1996; Díaz et al., 2013; Faoro and Orth, 2011; Ramírez and Davenport, 2013). In a comparison of the foraging behavior of honey bees in pear and apple orchards, Díaz et al. (2013) reported that these insects establish species-specific olfactory memories and adjust them to the fluctuations in the resource availability. They observed intense pollen foraging activity during pear flowering, followed by a reduced activity for pollen and increased activity for nectar with the start of apple flowering (Díaz et al., 2013). The differences observed in the present study for the pear and apple floral resources might explain this behavior.

Another consideration is the accessibility of the floral resources. The pear nectar and pollen are easily available for honey bees, while the nectar is less accessible in apple flowers, as the nectaries are located within the hypanthium (Farkas and Zajácz, 2007; Stern et al., 2007). For apple, the majority of the honey bees that gathered nectar did so without touching the anthers or stigmas, due to 'side-working' (Farkas and Zajácz, 2007; Martins et al., 2015; Ramírez and Davenport, 2013; Stern et al., 2007). As a consequence, pollen-collecting bees are more efficient for pollination of apple flowers (Ramírez and Davenport, 2013). These characteristics in terms of the accessibility and insect behavior might explain why more honey bees are required for apple (20 to 25 bees per tree per minute) than for pear (10 to 15 bees per tree per minute) pollination (Mayer et al., 1986, 1990). However, greater pollinator functional diversity can lead to improved seed set and many wild bee species exhibit functional traits that complement honey bee pollination in apple (Blitzer et al., 2016; Martins et al., 2015). For example, andrenid bees and bumble bees nearly always made contact with the stigma when visiting apple flowers and bumble bees can be active in temporal and environmental conditions unfavorable for both honey bees and andrenids (Martins et al., 2015).

4.2. Valuable fruit production depends on efficient insect pollination in pear and apple trees

Based on the observed flower visitation rates and the mean number of flowers per tree, we estimate that for pear and apple trees in this orchard, there were about 5 ± 3 and 17 ± 2 insect visits per tree per minute. This is lower than that given in the recommendations for valuable pollination (Mayer et al., 1986, 1990). However, the fruit set of most of the cultivars was higher than the 10% required for a full economically viable crop (Benedek et al., 2001; Sheffield, 2014). The observed seed sets were also the same as those obtained after compatible hand pollination (data not shown), which indicates that the insect pollination was not limiting during our observations and that the fruit production was valuable.

Despite high pollen grain deposits and pollen-tube growth, fruit set and seed set varied among cultivars. These differences could be related to physiological constraints or incompatibility (Michotte-van der Aa and Jacquemart, 2003; Monzón et al., 2004; Quinet and Jacquemart, 2015; Sanzol and Herrero, 2001). Low stigmatic receptivity, slow pollen-tube growth in the style, and low ovule viability can indeed limit pollination and fertilization (Jacquemart, 2007; Monzón et al., 2004; Sanzol and Herrero, 2001; Sanzol et al., 2003). In pears, the high fruit set and low seed set in 'Conférence' might be due to the development of parthenocarpic fruits (Jacquemart and Michotte-van der Aa, 2003; Quinet and Jacquemart, 2015; Warnier, 2000). In apples, 'Jonagored' was only semi-compatible with the other apple cultivars (Broothaerts et al., 2004), which might explain its low seed set.

The relative abundance of the different cultivars within the orchard could also influence the fruit and seed sets. Several pear and apple cultivars were mixed in our orchard which allowed us to compare visitation rates and floral resources independently of landscape and/or weather context. However, this orchard design favored cross-pollination and may overestimate the fruit and seed sets compared to many modern orchards. Most often only one or two pollinizer varieties are present in the intensive orchards in addition to the focal cultivar and they alternate every two to four-five rows of the focal cultivar (Blitzer et al., 2016; Delaplane et al., 2000; Díaz et al., 2013; Garratt et al., 2014; Monzón et al., 2004; Stern et al., 2004). Moreover, density of bee hives in our orchard could exceed this of most intensive orchards although it was within the recommended range (1–5 hives/ha; Stern et al., 2007). Pollen transfer limitation and subsequent reduced seed set could be observed in orchards despite the presence of hives (Garratt et al., 2014; Martins et al., 2015; Viana et al., 2014). Martins et al. (2015) showed that seed set was negatively predicted by the orchard area and by the distance from surrounding meadows. Forests and meadows in the vicinity of the orchards could provide foraging and nesting resources for pollinating insects before and after the fruit tree flowering (Blitzer et al., 2016; Martins et al., 2015). Both, successive introduction of honey bee hives during the flowering period and increase of pollinator diversity were shown to enhance fruit and seed sets in pear and apple orchards (Blitzer et al., 2016; Calzoni and Speranza, 1996; Garibaldi et al., 2013; Gupta, 2005; Martins et al., 2015; Stern et al., 2007; Viana et al., 2014; Rader et al., 2015).

4.3. Conclusion: management implications

Here we report that insect foraging behavior in pear and apple orchards can be mainly explained by the quality of the floral resources. Pear flowers are mainly visited for their pollen, as they produce pollen that is rich in polypeptides, amino acids, and phytosterols. Apple flowers produce sweeter nectar than pear flowers, and they are more visited for nectar collection. Moreover, within these species, the quantity and quality of the floral resources can differ among the cultivars, which highlights the differences in the visitation rates. It would thus be of interest to investigate floral resource quality, mainly pollen, to determine the quality of cultivars as pollinizer and to improve the floral resource attractiveness of cultivars in breeding programs. Moreover, further investigations are still needed to understand the impact of intraspecific differences in floral resources among cultivars on pollination efficiency of managed and unmanaged bees. Both pear and apple trees are early and mass flowering species that could constitute valuable resources at the stage of emergence of wild bees and colony foundation for social insects (Moquet et al., 2015). However, suitable foraging resources prior and following pear and apple trees must be

available in the vicinity of the orchards. Diversified floral resources are needed in bee diets to meet their nutritional requirements (Moquet et al., 2015; Somme et al., 2014). Maintaining diverse floral resources in abundance throughout the season in and around orchards would help maintain bee fauna after the mass flowering. Management programs at the orchard and landscape scales were proposed to enhance the diversity and abundance of wild pollinators (Blitzer et al., 2016; Martins et al., 2015). They could be interesting to maintain wild bee populations in the context of pollinator decline (Garibaldi et al., 2011) and to increase pollination service in the orchards and subsequently improve fruit production (Blitzer et al., 2016; Garibaldi et al., 2013; Martins et al., 2015).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eja.2016.04.001.

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