



# The impact of pollen quality on the sensitivity of bumblebees to pesticides

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## ABSTRACT

With 2000 species currently recorded in Europe, bees are a highly diversified and efficient group of pollinating insects. Their decline could therefore lead to a risk for ecosystems functioning and crop yields. The drivers of this decline have been well documented in Europe and involve multiple factors such as pesticides, pathogens, poor nutrition, climate change, and their respective interactions. For pesticides, there is a lack of information concerning the impacts of exposure when the bees have access to pollen diet of different nutritional quality (e.g. variation in sterol and protein content). We performed bioassays on nine groups consisting of 10 *Bombus terrestris* microcolonies (i.e. queen less colonies) each in a fully crossed experiment including controls without pesticide, two different doses of neonicotinoid imidacloprid (2 and 20 µg/L) and three pollen diets (i.e. *Salix* mix, cellulose-diluted *Salix* mix and cellulose-diluted *Salix* mix enriched with soy protein). All colonies were fed *ad libitum* with syrup and pollen. We measured the collection of pollen and syrup, the brood mass and the pollen efficacy (ratio of brood mass/pollen) at the end of the experiment. All these variables were affected by the imidacloprid doses, especially pollen efficacy, which was two and 10 times lower than the control for the 2 and 20 µg/L treatments, respectively. As expected, pollen efficacy was also affected by the pollen diet. However, when exposed to imidacloprid, the three different pollen diets did not affect micro-colonies performances. Overall, we show that diet quality does not affect sensitivity to imidacloprid exposure. It seems therefore that the negative effects of imidacloprid exposure cannot be compensated for by a pollen diet of high quality.

## 1. Introduction

With more than 85% of plant species requiring biotic pollination for their own reproduction (Ollerton et al., 2011), pollinators are not only necessary to sustain wild plant communities but they also provide an important ecosystem service in doing so (Potts et al., 2016). Pollination of plants is driven by multiple animal groups, but bees are the most important one (Potts et al., 2016) because of their diversity (20,000 species), their ubiquity and their life cycle relying almost exclusively on pollen and nectar consumption (Michener, 2007). Several bee species, including crop pollinating species, are currently in decline (Carvalho et al., 2013; Goulson et al., 2015). Bumblebees (*Bombus*) are a group of important pollinators, but almost one third of the species appear to be declining (Arbetman et al., 2017). The reasons for these declines have been well documented in Europe, indicating different factors like the loss of habitat (e.g. Persson et al., 2015; Vray et al., 2019), invasive species (e.g. Stout and Morales, 2009), parasites (e.g. Williams et al., 2008), pathogens (e.g. Ravoet et al., 2014), global warming (e.g. Kerr et al., 2015) and agricultural intensification (e.g. Winfree, 2010). Agricultural intensification has occurred most notably in the past 70

years with the increasing demand for food (Matson et al., 1997) and has relied on the introduction of productive crop varieties, intensive mechanisation and the use of various agrochemical products, including pesticides.

Pesticides can occur in trace levels in the nectar and pollen of crop plants and therefore be consumed by bees or brought back to the nest (Blacquière et al., 2012). By dissolving in soil water, molecules and their metabolites can be persistent and can be found at surprisingly high concentrations in the pollen and nectar of wild flowers around a treated crop (Krupke et al., 2012). It has been shown that pesticides can impact bumblebee fitness for several reproductive traits (e.g. colony growth, ovarian development, queen production) under laboratory conditions (Mussen et al., 2004; Whitehorn et al., 2012; Baron et al., 2014) as well as foraging efficiency under field conditions (Feltham et al., 2014; Gill and Raine, 2014; Stanley et al., 2016). Bumblebee learning, memory and odour perception can also be negatively affected by agrochemicals (Stanley et al., 2015). Moreover, Mommaerts et al. (2010) showed that bees can be more sensitive to pesticides in semi-field conditions than in laboratory conditions, partly explained by the distance to reach resources. Among pesticides, neonicotinoids are systemic and target a

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broad spectrum of piercing and sucking insect pests by acting as nicotinic acetylcholine receptor (nAChR) agonists in the central nervous system (Tomizawa and Casida, 2003; Elbert et al., 2008). The impacts of this molecule have been relatively well studied on bees (Cresswell et al., 2012; Laycock et al., 2012; Gill and Raine, 2014; Phelps et al., 2018; Wu-Smart and Spivak, 2018). Binding to nAChRs impairs normal cognitive and a suite of behavioural functions in bees including most of those explained above (Decourtye et al., 2003; Mommaerts et al., 2010; Feltham et al., 2014; Rundlöf et al., 2015; Stanley et al., 2015, 2016; Wu-Smart and Spivak, 2018).

Besides the use of pesticides, agricultural intensification also leads to the loss of botanically diverse landscapes through their transformation into large monocultures which can also impact bees through nutritional stress (Persson et al., 2015). This lack of diversity can impair the access to a balanced diet (balanced profiles of amino-acid, sterols and protein:lipid ratio) and impact offspring number, colonies size, mortality and immunity (Tasei and Aupinel, 2008a; Vanderplanck et al., 2014, 2018; Vaudo et al., 2016; Moerman et al., 2017; Roger et al., 2017). It could also imply a loss of a favorite host-plant and hence induce starvation and developmental delay in bumblebee colonies (Yoon et al., 2005; Sutcliffe and Plowright, 2008).

Interactive effects between nutrition and pesticide exposure have rarely been assessed for bees, especially regarding reproductive traits (Schmehl et al., 2014; Leza et al., 2018). Nonetheless, it is known that a poor diet can affect the transcription level of the CYP450 and the Glutathione-S-transferase activity (Alaux et al., 2011; Di Pasquale et al., 2013; Corby-Harris et al., 2014), both involved in detoxification pathways (Berenbaum and Johnson, 2015). Nutritional stress could therefore lead to a higher sensitivity to agrochemicals. Moreover, pesticides can impact nutritional intake itself by impairing locomotion (Cresswell et al., 2012), disorientation (Han et al., 2010) and foraging abilities (i.e. proboscis extension) (Phelps et al., 2018). Overall, it seems crucial to better investigate how pollen quality could reduce or increase the impact of pesticide exposure on bee fitness.

To address these knowledge gaps, we used *Bombus terrestris* as study model and tested the effects of pesticides on colonies fed on three pollen diets showing different chemical qualities: (i) a beneficial pollen diet (*Salix* mix) as control, (ii) a 30% diluted *Salix* mix diet to test the impact of a low-quality diet, and (iii) 30% diluted *Salix* mix diet enriched with soy protein to assess the implication of protein in bumblebees resistance to pesticides. We considered three treatments of chronic exposure to pesticide: (i) control (no pesticide), (ii) field realistic dose of imidacloprid (2 µg/L), and (iii) high exposure dose of imidacloprid (20 µg/L). For each colony we monitored the rate of resource collection (the quantity of pollen and syrup collected) and the rate of brood development and pollen efficacy (larval mass developed per gram of consumed pollen). Regarding the importance of nutrition for bee development and the negative impact of imidacloprid on bee health, we can expect that

both a poor diet and imidacloprid exposure will reduce micro-colonies performances. Moreover, these two factors might act synergistically with a poor diet. That could reduce the detoxification abilities of bees and then strengthen the negative impact of imidacloprid.

## 2. Material and methods

### 2.1. Study species

Bumblebees were provided by Biobest NV (Westerlo, Belgium). A total of five queen-right colonies of 100 *Bombus terrestris* workers were used to build up 90 queen-less micro-colonies of five workers in plastic boxes (8\*16\*16 cm). This number of individuals per micro-colony has been optimized during previous bioassays (Moerman et al., 2016; Roger et al., 2017; Vanderplanck et al., 2018) and has been shown to be the most favourable for male offspring production (Gradish et al., 2013). Moreover, using more workers can dilute the brood tending responsibilities across more individuals, inducing a microclimate temperature elevation (Klinger et al., 2019). A hierarchical system occurred quickly in micro-colonies with a worker exerting its dominance on the others and laying haploid male eggs (Tasei and Aupinel, 2008b). For each experimental condition, a total of 10 micro-colonies were used with two micro-colonies coming from one queen-right colony to avoid any colony-related bias. All micro-colonies were maintained in the same room in constant darkness with a relative humidity of 60–65%. They were manipulated under red light to minimize disturbance (Sadd, 2011) for a period of 28 days.

### 2.2. Pollen diet composition

*Salix* pollen mix was used as control diet as it is well known to be highly suitable for *B. terrestris* (Tasei and Aupinel, 2008a; Moerman et al., 2016; Vanderplanck et al., 2018). *Salix* content of this mix was around 85%. To reduce pollen quality, we artificially diluted it by mixing willow pollen mix with cellulose at a ratio of pollen/cellulose 10:3. We used cellulose because it does not appear to be detected by bumblebees (Mapalad et al., 2008; Konzmann and Lunau, 2014). We then considered the same diluted diet enriched with soy protein to recover the initial protein content so that proteins were the only macronutrients in high concentration (other nutrients still diluted with cellulose). Such use of soy protein has already been chosen in case of protein enriched diets experiments on bees (Roulston and Cane, 2002; Alqarni, 2006; De Jong et al., 2009). Overall, bumblebees were exposed to three different diets to assess nutritional stress: (i) a *Salix* mix diet as control with a protein content of 24% (Fig. 1A); (ii) a diluted *Salix* mix diet with a protein content of 17% (Fig. 1B), and (iii) a diluted *Salix* mix diet enriched with soy protein to return to a 24% protein content (Fig. 1C). Protein content was evaluated according to micro Kjeldahl

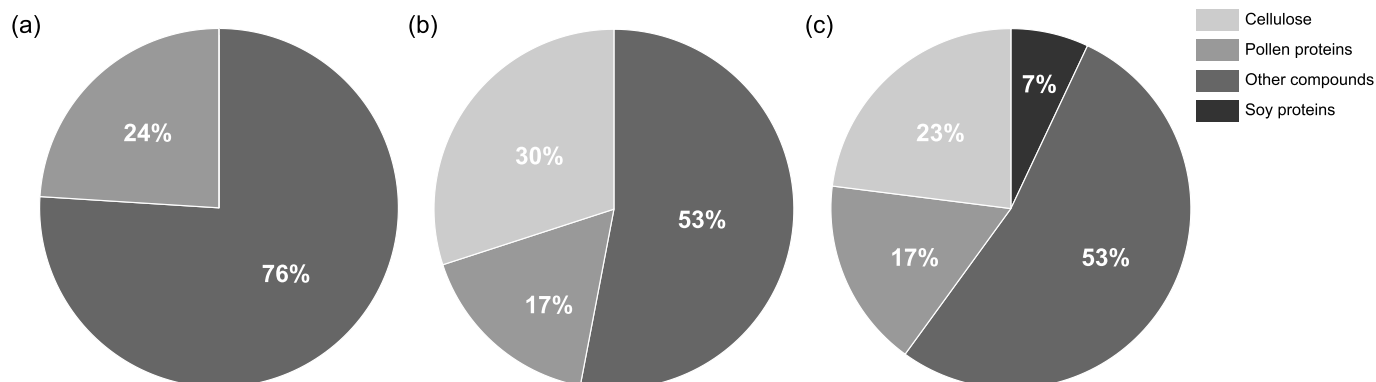
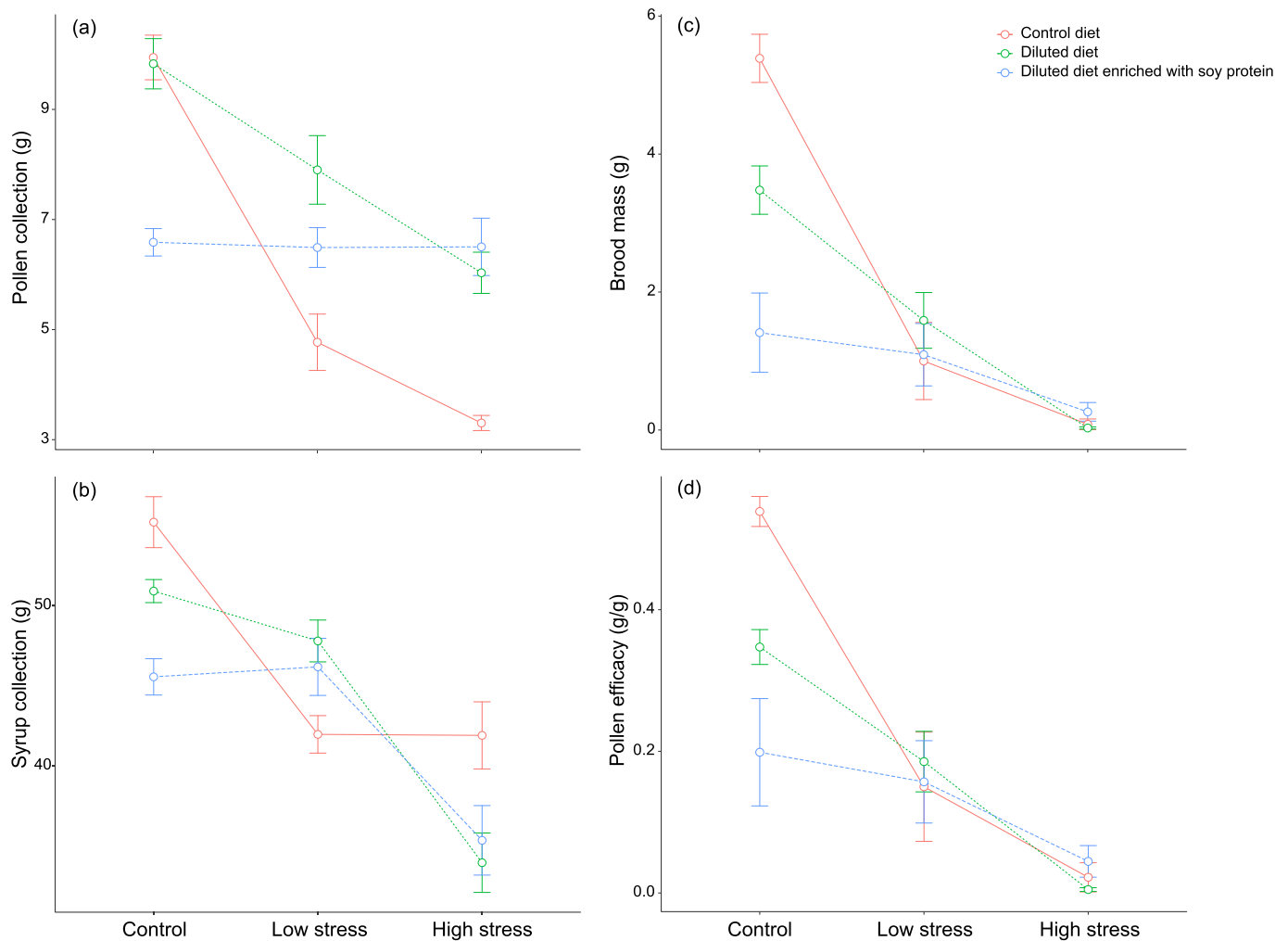


Fig. 1. Composition of pollen diets. Protein content was determined by micro Kjeldahl method. (a) *Salix* mix diet, (b) diluted *Salix* mix diet and (c) diluted *Salix* mix diet enriched with soy protein.



**Fig. 2. Resource collection and micro-colony development.** Pollen collection (a), syrup collection (b), brood mass (c) and pollen efficacy (d) of micro-colonies exposed to different levels of stresses (mean  $\pm$  SE). For statistical details see Table S1. Each treatment has 10 replicates.

methods using the protocol from Kirk (1950) and Conti et al. (2016). A minimum quantity of 500 mg per sample was digested for 4 h at 400 °C using 20 mL of 98% sulphuric acid and a single 5 g Kjeldahl tablet (catalyst containing sodium sulphate, potassium sulphate and copper sulphate). After alkalisation with 50% NaOH solution, the digest was steam-distilled and condensed in 2% boric acid solution. The obtained solution was then titrated with sulphuric acid 0.5 N for nitrogen determination. The protein content was computed using 6.25 as conversion factor as it is the most commonly used (Conti et al., 2016).

Pollen diets were provided to the micro-colonies as candies, a mixture of pollen loads and syrup (0.17 mL of syrup per gram of pollen). Pollen loads were purchased from the company "Pollenergie" (France), cellulose powder from Sigma-Aldrich, and soy protein from Purasana® (Belgium). Pollen loads and soy proteins are sold as organic nutrition complement (i.e. free of pesticides). Prior to the experiment, blends of pollen loads were mixed with sugar syrup to obtain consistent candies stored at  $-20$  °C. New pollen candies were provided every two days, while the previous ones were removed before drying or decaying and weighed to assess the pollen collection.

### 2.3. Pesticide treatments

We used a commercial solution of imidacloprid (Confidor 200 SL®, Bayer Crop Science), a neonicotinoid pesticide. Imidacloprid was administered chronically for 28 days in syrup feeders at 2 or 20  $\mu\text{g/L}$  while syrup used to prepare pollen candies remained pesticide free. The

2  $\mu\text{g/L}$  concentration is based on residues that have been found in pollen and nectar in the field (Bonmatin et al., 2003, 2005; Decourtye et al., 2003; Chauzat et al., 2006; Krupke et al., 2012) whereas the 20  $\mu\text{g/L}$  concentration is less realistic but can nonetheless be observed in extreme conditions (Krupke et al., 2012). To reach these concentrations, pesticide was diluted multiple times first in water, then in sugar syrup. Solutions were given to the bees in a 100 mL glass container with a capillary. Control and contaminated syrup were replaced every four days to avoid moistures and degradation and weighed to assess sugar and pesticide consumption. Each nutritional condition previously described was crossed with the two pesticide treatments and the control, which led to a total of nine experimental conditions with 10 micro-colonies per condition.

### 2.4. Assessed parameters

To estimate performance and development of bumblebee micro-colonies, several parameters were evaluated: (i) total pollen and syrup collections, which can impact brood production and development (e.g. Plowright et al., 2008; Sutcliffe and Plowright, 2008); (ii) colony growth after 28 days of development [i.e. mass of individuals from all brood stages (eggs, larvae, pupae, non-emerged and emerged males)] (Vanderplanck et al., 2014, 2018). For each micro-colony, all the weighted parameters were divided by the total mass of the five workers to standardize the results and avoid potential effect of worker activities related to their size (i.e. consumption and brood care). Additionally, we

calculated the pollen efficacy as the mass of total offspring divided by total pollen collection to estimate the colony performance.

### 2.5. Statistics

We performed comparative analyses of the colony performance and feeding behaviour using R version 3.6.0 (R Core Team, 2017). Statistical analyses using two-way crossed analyses of variance (Two-Way crossed ANOVA) were conducted to evaluate the effect of diet and pesticide stress as well as their interaction. Since it is a parametric test, homoscedasticity (Bartlett test) and normality of the residuals (Shapiro test) were checked prior to the analyses. When violation occurred, data were log- or rank-transformed to normality of residuals (“*rnttransform*” function, R-package “GenABEL”) prior to the test. Multiple pairwise comparisons were conducted using Tukey HSD tests when ANOVA detected significant difference among host-plants ( $P < 0.05$ ).

## 3. Results

Our results show that imidacloprid exposure significantly impacted the development and the feeding behaviour of micro-colonies, but that these effects depended on the diet treatment (i.e. significant interactive effects, Fig. 2 and Table S1).

Pesticide exposure significantly reduced the brood development (Fig. 2c). This negative effect was not accentuated by the imidacloprid dose since no significant difference has been detected between low and high exposure (i.e. 2 and 20 µg/L of imidacloprid, respectively), regardless to the diet (Table S1). For micro-colonies fed on the control diet, the brood development significantly decreased when exposed to low pesticide dose, and remained low for the highest exposure. In the same way, brood growth significantly decreased for micro-colonies fed on the diluted diet but only between the treatment without pesticide and the high stress (Table S1). No effect of pesticide has been detected in micro-colonies fed on the diluted diet enriched with soy protein, the brood development being overall reduced for this treatment (Fig. 2c).

Regarding feeding behaviour, micro-colonies showed decreases in both pollen and syrup collection along with the pesticide exposure (Fig. 2a and b). Micro-colonies fed on control diet showed declines in both pollen and syrup collection when exposed to a low pesticide dose compared to control without pesticide, the effect on pollen collection being much more accentuated for micro-colonies exposed to the high imidacloprid dose (Fig. 2a and b). As for brood development, the negative effect was less pronounced for the other diets. For the diluted diet, the effect on both pollen and syrup collection was only significant when bees were exposed to a high pesticide dose compared to control without pesticide (Fig. 2b), while a significant effect was only detected for pollen collection between control without pesticide and high pesticide exposure in micro-colonies fed on the diluted diet enriched with soy protein (Fig. 2b, Table S1).

Overall, imidacloprid exposure reduced the pollen efficacy of the diet (Fig. 2d, Table S1). A significant effect was detected between control and exposed micro-colonies (i.e. both 2 and 20 µg/L of imidacloprid) when fed on the control diet; as well as between control without pesticide and highly exposed micro-colonies (i.e. 20 µg/L of imidacloprid) when fed on the diluted diet. Micro-colonies fed on the diluted diet enriched with soy protein followed the same trends but the effect was not significant regardless to the intensity of the pesticide exposure (Fig. 2d; Table S1).

## 4. Discussion

Our results highlight that imidacloprid exposure can decrease both colony growth and resource collection, such impacts being not reduced by nutrient- or protein-rich diets. Especially, we found a strong effect of imidacloprid exposure on pollen efficacy, with the larval production per gram of collected pollen being at least four times lower in micro-

colonies exposed to pesticide compared to the control ones. Beside this effect on pollen efficacy, pesticide exposure also decreased the pollen collection in our experiment. These negative effects may be accentuated in field conditions as pesticide exposure may impair orientation and learning (Blacquière et al., 2012; Tsvetkov et al., 2017), impeding then even more the resource collection. Moreover, other chemicals such as residues from herbicides and fungicides used by farmers might display synergetic effects with the imidacloprid, strengthening its negative impact. Indeed, although the outdoor use of the three major neonicotinoid compounds (clothianidin, imidacloprid, thiamethoxam) has been banned in Europe (European Commission, 2018), there are still many molecules that require a complete risk assessment, including both new and old fungicides and herbicides.

The effects of exposure to agrochemicals on bee health have been extensively studied, particularly for neonicotinoids (Blacquière et al., 2012; Laycock et al., 2012; Woodcock et al., 2016; Stanley et al., 2015; Tsvetkov et al., 2017; Leza et al., 2018; Wu-Smart and Spivak, 2018). Previous studies have shown that neonicotinoids can affect bumblebee mobility, inducing symptoms such as knockdown, trembling, uncoordinated movements, hyperactivity and tremors (Lambin et al., 2001; Nauen et al., 2001; Medrzycki et al., 2003; Colin et al., 2004; Suchail et al., 2001). It can also affect nutritional intake by impairing the proboscis extension reflex (Decourtye et al., 2005, 2003), learning, memory or taste/odour perception (Stanley et al., 2015; Hesselbach and Scheiner, 2018). These findings could explain the reduction in pollen collection by exposed bumblebees in our study. Also, it is known that bumblebees tend to be more attracted by food containing neonicotinoids but they also tend to consume it in fewer quantities compared to an untreated diet (Kessler et al., 2016). Regarding the colony development parameters (i.e. brood mass), our results may suggest that when exposed to imidacloprid, the dominant worker had a lower egg-laying frequency than in control colonies. However, Laycock et al. (2012) showed that exposure to imidacloprid does not prevent ovary development. To lay eggs, *B. terrestris* workers need a social stimulus to initiate brood production (Amsalem et al., 2009). It is therefore possible that imidacloprid has an impact on social interactions, leading to an indirect disruption of the egg laying mechanism.

Regarding the diet quality and its potential impact on the sensitivity of bumblebees to pesticides, our result suggest that bumblebees were probably not able to detect cellulose in pollen and to adapt their foraging behaviour accordingly, which corroborates previous studies (Mapalad et al., 2008; Konzmann and Lunau, 2014). Diluted pollen was then collected in the same quantities as raw pollen in control colonies, which directly impacted the colony growth as pollen dilution reduced the density of all nutrients by 30% and as bees require these nutrients for their development (Brodschneider and Crailsheim, 2010). Interestingly, decreases in brood mass were close to 30% compared to control diet, which corresponds to the proportion of cellulose added to the diet for dilution. Note that the negative effects of diluted diet are not necessarily due exclusively to nutrient dilution, but could also be due to potential cellulose impacts on larval development (e.g. water depriving or constipation). During our experiment, the addition of soy protein to the diluted diet failed to offset the effects of a low-quality diet. Observed results were even worse than those obtained using diluted pollen alone, which was unexpected. This artificial diet may have reduced feeding due to an excess of particular amino acids, or the larvae may not have been able to effectively digest the soy proteins. Moreover the ratio between proteins and lipids has been shown to play an important role on bumblebee foraging strategies and floral preferences (Vaudou et al., 2016). By artificially modifying the protein content of pollen, it is possible that this macro-nutrient ratio became inadequate for bumblebees. These hypotheses have to be supported by further experiments using other protein sources and/or different pollen types with a naturally lower protein content.



#### 4.1. Conclusion

Our findings highlight that a realistic dose of imidacloprid (2 µg/L) can strongly affect pollen collection as well as the reproduction parameters of bumblebees, which is in line with previous observations (Laycock et al., 2012; Feltham et al., 2014; Gill and Raine, 2014). Unfortunately, it appears that bumblebees do not benefit from the advantages of a nutrient-rich diet when exposed to imidacloprid, suggesting that the impact of imidacloprid cannot be mitigated by access to a high-quality diet. This could originate from a difference in nutrient allocation according to the environmental stress (i.e. pesticide exposure). While the nutrients from a high-quality diet could be used to increase larval production in control condition, they might rather be used for adult immunity and survival when the colony is exposed to an environmental stress such as pesticide exposure. However, the difference in resource allocation might be less visible for a diet poorer in nutrients as brood production would be reduced even in control conditions.

While current mitigation strategies are mainly oriented to provide additional floral resources (e.g. floral strip) (Feltham et al., 2015), our results show that such practices are far from being sufficient and reinforce the necessity of a total ban of pesticide in the world. Currently Europe has restricted any outdoor use of imidacloprid as well as two other neonicotinoids (European Commission, 2018). Unfortunately, new pesticides with a similar mode of action are already emerging (i.e. Sulfoxaflor), which raises the alarm. Reduction of the use of phytochemicals as well as quick complete risk assessments before a wide global distribution of new agrochemicals are actually key to preserve the biodiversity.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.actao.2020.103552>.

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