Interaction between the alien plant *Impatiens glandulifera* Royle (Balsaminaceae) and native visitor communities

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Abstract

Plant-bee interaction forms one of the most fascinating examples of co-evolution in the living world. Most bee species rely exclusively on flowering plants through pollen and nectar foraging, allowing their nutrition and the one of their larvae. Bee contacts with flowers generally lead to pollen transport, resulting in the fertilization and therefore in the perpetuation of the majority of entomophilous plant species. However, this mutualism is now affected by various new stresses. Amongst them, alien organisms interfering in the native plant-bee interactions have passioned conservation biologists for decades. In this work are presented the impacts of an invasive plant from Asia, the Himalayan Balsam (Impatiens glandulifera Royle, Balsaminaceae) on native visitor communities in Belgium. Following previous observations showing that parasite prevalence in the bumblebee Bombus pascuorum was lower in Balsam-invaded sites, three hypotheses were tested: (i) the detected parasite difference could be due to different floral visitor communities (e.g. differences in parasite-sharing bees such as Apis mellifera), (ii) the Balsam could present a panel of medicinal compounds in its floral resources, potentially curing bumblebees and (iii) ecosystemic conditions prior to the Balsam’s blooming (e.g. floral assemblage and pollen diets) could have impacted bumblebee parasitism. First, sites invaded by the alien were compared to non-invaded sites, showing that the panel of floral visitors is significantly affected by Impatiens glandulifera, but that parasite-sharing organisms were not likely to explain any difference in parasite transmission regarding site type. A focus on Bombus pascuorum showed that this species has adopted the plant as part of its pollen diet in invaded sites, with more than 23% of its pollen loads being pollen grains from the invader. Moreover, chemical analyses have revealed that pollen from I. glandulifera contained a rich panel of polyphenols, including the well-known flavonoid amelopsin, widely reported in literature for its medicinal effects. Finally, through morphological analyses of wings, Bombus pascuorum from invaded and non-invaded sites were compared and did not show significant difference that could have been due to different pre-imago diets. Therefore, our results suggest that the rich polyphenolic profile of the Balsam’s pollen could positively affect the health of the native bumblebee Bombus pascuorum in alien-invaded sites by decreasing its pathogen prevalence.

Key words: Impatiens glandulifera, invasive species, bumblebee, parasite prevalence, medicinal plants, polyphenols
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# Table of contents

I. Introduction................................................................................................................................. 6
   1. Bees: diversity and ecology..................................................................................................... 6
      1.1. Generalities..................................................................................................................... 6
      1.2. Bumblebees.................................................................................................................... 7
         1.2.1. Generalities.............................................................................................................. 7
         1.2.2. Bumblebee parasites............................................................................................... 8
         1.2.3. Floral choices.......................................................................................................... 9
      1.3. Quality of the floral resources......................................................................................... 10
         1.3.1. Pollen and nectar...................................................................................................... 10
         1.3.2. Non-nutritive metabolites........................................................................................ 12
      1.4. A case of study for secondary metabolites: antioxidants............................................. 14
         1.4.1. Generalities.............................................................................................................. 14
         1.4.2. Roles of flavonoids in plants.................................................................................... 15
         1.4.3. Impacts of flavonoids on insects.............................................................................. 15
         1.4.4. Effects of polyphenols on parasitism....................................................................... 16
   2. Invasive plant species.............................................................................................................. 18
      2.1. Generalities..................................................................................................................... 18
      2.2. The case of *Impatiens glandulifera*........................................................................... 18
II. Background and objectives........................................................................................................ 20
III. Material and methods............................................................................................................ 23
   1. Plant-visitor associations....................................................................................................... 23
      1.1. Plant visitors................................................................................................................... 23
      1.2. Palynology...................................................................................................................... 25
   2. Analytical chemistry on floral resources from *I. glandulifera*........................................... 27
   3. Geometric morphometrics on wings from *B. pascuorum* workers.................................... 29
   4. Data analyses.......................................................................................................................... 30
      4.1. Plant-visitor associations............................................................................................... 30
      4.2. Wing morphometrics..................................................................................................... 31
4.2.1. Wing size........................................................................................................31
4.2.2. Wing shape....................................................................................................31

IV. Results..................................................................................................................32
   1. Floral visitor communities in invaded and non-invaded sites...........................32
      1.1. Comparison of visitor communities.........................................................32
      1.2. Palynology on the pollen loads from Bombus pascuorum workers........34
   2. Polyphenolic composition of pollen and nectar from Impatiens glandulifera.....35
   3. Morphological analyses on Bombus pascuorum wings....................................37

V. Discussion.............................................................................................................38
   1. Impacts of Impatiens glandulifera on the floral visitor communities............38
   2. Bee community and parasite community.......................................................40
   3. Secondary metabolites in the pollen of Impatiens glandulifera.....................41
   4. Habitat quality before the blooming of Impatiens glandulifera.....................43

VI. Conclusion..........................................................................................................44

VII. Perspectives......................................................................................................46

VIII. References.......................................................................................................48

IX. Appendices.........................................................................................................67
I. Introduction

1. Bees: diversity and ecology

1.1. Generalities

Sometime in the mid Cretaceous (~120 megaannum) diverged from predatory apoid wasps the monophyletic group Anthophila, formed by the widely known bees (Michez et al. 2012; Michener, 2007). More than 20,000 species are described worldwide, with more than 2000 of them being reported in Europe (Nieto et al. 2014). Their emergence and the following explosion of their diversity has been linked with the diversification of angiosperms, the latter represented by more than 250,000 described species (Grimaldi, 1999; Soltis and Soltis, 2004). Similar morphological characters unite all bee species into a group: the posterior part of the thoracic pronotal lobes are separated from the tegulae, and a pair of pronotal processes encircle (at least in part) the thorax behind the front coxae (Michener, 2000). Morphologies vary widely among families, tribes, genera and even species (Fig. 1) allowing most of the time their identification to the species level without the use of molecular data.

![Bee morphologies](Image)

Figure 1. Bee morphology varies widely among families, tribes, genera and species, as shown here with (a) Anthophora plagiata, (b) Eucera dimidiata and the two cleptoparasitic bees (c) Dioxyx cincta and (d) Melecta sp. Photos by N. Vereecken (scales not provided in the original pictures).
Most bee species present a solitary lifestyle, i.e. without any contact between generations. Some bee families such as Andrenidae and Melittidae only present this level of sociality. Nevertheless, in Apidae (Michener, 2007) and Halictidae (Timmerman and Kuhlmann, 2008), social interactions between individuals from the same species involving a collaboration between at least two individuals living in a common nest are reported (Pouvreau, 2004). The feeding behavior of bees is generally correlated with their level of sociality: while many polylectic bees (i.e. bees foraging on a wide panel of plant species) are social, most oligolectic and monolectic ones (i.e. specialist bees) present a solitary lifestyle (Cane and Sipes, 2006).

1.2. Bumblebees

1.2.1. Generalities

Bumblebees (Hymenoptera: Apoidea: genus *Bombus*), phylogenetically closely related to the well-known honeybee, are bees represented by more than 250 species worldwide (Williams, 2008). They form a critically important group of pollinators, providing ecosystem services both for agricultural crops and natural environments (Klein *et al*. 2007). Since the 1970’s, bumblebee decline has been pointed out in Europe (Peters, 1972; Rasmont and Mersch, 1998), then later in North and South America (Cameron *et al*. 2011; Arbetman *et al*. 2013) and even in China (Xie *et al*. 2008). The major hypotheses proposed to explain this decline are (i) habitat fragmentation (e.g. Hatten *et al*. 2013), (ii) urbanization (e.g. Ahrné *et al*. 2009) (iii) the shortage of flower resources in their environment (e.g. Goulson *et al*. 2005), (iv) the overgrazing of their habitat by cattle (e.g. Özbek, 1995), (v) their widespread parasites and pathogens (e.g. Arbetman *et al*. 2013), (vi) vegetation displacement due to nitrogen deposition (Rasmont, 2008) and climate change (Rasmont *et al*. 2015). Pesticides may also play a key role in their decline but unfortunately, their impact remains more underestimated for bumblebees than for honeybees (Whitehorn *et al*. 2017).
1.2.2. Bumblebee parasites

Bumblebees in the wild can suffer from various parasites, their transmission being sometimes facilitated by heavily-infected bumblebees from commercial rearing. This transfer from reservoir populations to sympatric wild populations is known as pathogen spillover (Daszak et al. 2000), the latter potentially leading to rapid declines in the novel host populations (e.g. Haydon et al. 2006, for bumblebees see Meeus et al. 2011). Among those parasites are reported:

- **Crithidia bombi** – *C. bombi* (Kinetoplastida: Trypanosomatidae) is a hind gut parasite able to infect all casts in bumblebee colonies when ingested. Contamination can occur by a contact with a plant touched by an infected bee or in infected nests (Durrer and Schmid-Hempel, 1994) and queens were reported to sometimes harbor *C. bombi*, this infection not necessarily preventing the bee to found a colony. Impacts of *C. bombi* on bumblebee’s health were shown to be condition-dependent, with a higher mortality rate under unfavorable nutrition intake (Brown et al. 2003).

- **Apicystis bombi** – *A. bombi*, despite its specific epithet, is the name of a neogregarine parasite (Apicomplexa: Lipotrophidae) able to affect both *Bombus* spp. and *Apis mellifera* (Lipa and Triggiani, 1996). Known to infect nearly 20 *Bombus* species including commercial colonies (Meeus et al. 2011; Murray et al. 2013), its proliferation can lead to destructions of the bee fat bodies (Schmid-Hempel, 2001) and prevent queens to establish colonies, due to a higher mortality rate (Macfarlane et al. 1995; Rutrecht and Brown, 2008).

- **Nosema apis** – This parasite, reported early in Europe in the honeybee *Apis mellifera* (Zander 1909), is a microsporidium (Microsporida: Nosematidae) transmitted horizontally via spore ingestion, for instance when workers clean combs contaminated by infected faeces (Fries 1988, Fries et al. 1993). Infected workers of bees show a reduced lifespan, colonies present a decreased honey production and an increased winter mortality (Fries et al. 1984; Anderson and Giacon 1992). Nevertheless, *N. apis* may show lower virulence than its congeneric species *N. ceranae* (Paxton et al. 2007).
• **Nosema ceranae** - Until recently, *N. ceranae* (Microsporidia: Nosematidae) was reported as a parasite of *Apis cerana* (Fries et al. 1996) and *Apis mellifera* (Huang et al. 2007), before being also detected in South American bumblebees (Plischuk et al. 2009). The same author described that the macroscopical lesions of the ventriculus from infected bumblebees seem to show similarity with the ones of infected *Apis* spp. and touched all casts in studied bumblebee species. Experiments involving *N. ceranae* infecting honeybees versus healthy control show higher mortality in infected bees (Higes et al. 2007).

• **Nosema bombi** – This other fungus (Microsporidia: Nosematidae) was recognized since early in the 20th century (Fantham and Porter, 1914). While *N. apis* reproduces in the bee’s gut epithelium, *N. bombi* shows preference for Malpighian tubules (Fries et al. 2001) even if transmission process is identical, via bee faeces (Fantham and Porter, 1914; Fries, 1993; Mclvor and Malone, 1995). Impacts of the infection involve a higher production of males (Imhoof and Schmid-Hempel, 1999), paralysis of abdomen (MacFarlane et al. 1995) and a reduced lifespan (Bailey et Ball, 1991).

Bee parasites can benefit from the presence of shared resources through horizontal transmission (e.g. Durrer and Schmid Hempel, 1994; Graystock et al. 2015), parasite cells being deposited on flower surfaces and transported on forager surfaces (Cisarovsky and Schmid-Hempel, 2014). However, many studies in insects (e.g. Schaub and Loesch, 1989; Jaenike, Benway and Stevens, 1995) including bumblebees (Brown, Loosli and Schmid-Hempel, 2000; Brown et al. 2003) have shown that parasites may impact host mortality only under stressful conditions. For instance, pollen-starved bumblebees have been reported to support significantly smaller parasite populations than pollen-fed ones (Logan et al. 2004).

1.2.3. Floral choices

Plant communities can provide a large panel of flowers, all different in terms of appearance, quality and quantity of floral resources. While foraging however, a single bee
does not visit different plant species at random but tends to select one or a few species within a foraging bout. This behavior, generally termed floral constancy, has been widely studied by the past (see reviews by Menzel, 1985; Waser, 1986 and Chittka et al. 1999). This plastic foraging strategy (Kawaguchi et al. 2007) could probably reflect an ability from the bee to learn floral features, i.e. their odor, color and pattern and to use those traits as predictors of floral rewards (Menzel et al. 1993). Hence, this constancy is likely to benefit every co-visited plant species as it leads to a high pollen transfer between conspecifics.

In bumblebees, floral choices are reported to be led by floral quality, with a preferential foraging on pollen with high protein contents (Hanley et al. 2008; Leonhardt and Blüthgen, 2012; Robertson et al. 1999). While bumblebees have been shown to focus on highly concentrated resource-rich plant species, they have also been reported to switch to more rewarding alternate resources when they become available (Fontaine et al. 2008; Goulson and Darvill, 2004; Liao et al. 2011). In sympatric environments, generalist species were reported to present different foraging strategies, with *B. lapidarius* and *B. pascuorum* collecting high-quality pollen, while *B. terrestris* s.l. and *B. hypnorum* tended to enlarge their floral panel to less valuable pollen resources (Somme et al. 2015). Vaudo et al. (2016) hypothesized that bumblebees could favor a high Protein-Lipid ratio and therefore increasing their fitness. This discrimination among pollen species would be made possible via chemosensory receptors of the antennae (Ruedenauer et al. 2015), this hypothesis corroborating to other studies reporting a preference for pollen containing high levels of proteins (Leonhardt and Blüthgen, 2012; Kitaoka and Nieh, 2009).

1.3. Quality of the floral resources

1.3.1. Pollen and nectar

Pollen grains represent one of the main floral products used by bees for the development of adults and larvae. Their high protein concentration (up to 61% of dry mass, Roulston et al. 2000) often provides after digestion a large amount of amino acids used by bees to build their own structural and functional proteins. Protein concentration in pollen is positively correlated with imago reproduction (ovarian development and egg laying), larvae
development and feeding behavior (Sutcliffe and Plowright, 1990; Génissel et al. 2002; Human et al. 2007). Polypeptides were reported to improve immune function and cuticular melanization in insects (Lee et al. 2006, 2008). Pollen chemical quality is therefore of the utmost importance to produce a healthier offspring (Brodschneider and Crailsheim, 2010) and to reinforce tolerance to parasites (Szymas and Jedruszuk, 2003). Lipids, varying from 1 to 20% of dry mass in pollen, are reported to play roles various physiological processes from learning (Arien et al. 2015) to molting hormone production (Canavoso et al. 2001). A high sterol content in pollen is also reported to favor larvae development (Vanderplanck et al. 2014).

Since composition of pollen impacts bee development, some diets have been shown to be inadequate for some generalist bee species (De Groot, 1953; Sedivy et al. 2011; Haider et al. 2013). For those reasons, plants may be evolutionarily selected by pollinators for their pollen chemical composition by attracting them and potentially increasing their degree of fidelity (Vanderplanck et al. 2014).

Floral nectar, a sugar-rich aqueous solution produced by plants nectaries, is the main source of carbohydrates for pollinators. Possibly originating from the phloem sap (Fahn, 2000), floral nectar is a secretion related to plant reproduction, acting as an energetically adapted reward for pollinators (Heinrich, 1975). Nectar chemistry is dominated by three main carbohydrates: the disaccharide sucrose and the monosaccharides fructose and glucose. Their respective concentrations depend on the nectary invertase, an enzyme able to hydrolyze sucrose into fructose and glucose before or after nectar is secreted (Pate et al. 1985). In most nectars, a phenomenon of partial hydrolysis is responsible for the mixed sugar composition (Baker and Baker, 1983). Unusual monosaccharides, disaccharides and more rarely oligosaccharides can be present in nectar in trace amounts, passing untransformed from phloem to nectaries (Jackson and Nicolson, 2002). Total concentrations in sugars generally range from 7 to 70% w/w among plant species but vary widely due to ambient humidity and temperature (Corbet et al. 1979) and nectar resorption (Búrzquez and Corbet, 1991).
1.3.2. Non-nutritive metabolites

Plant tissues accumulate myriads of molecules mainly involved in plant signalization and defense against herbivores, bacteria and fungi (Schoonhoven et al. 2005; Agrawal and Weber, 2015). A wide variety of them, synthesized from primary metabolites, may confer protection against stresses from the environment (i.e. salinity, alkalinity, drought, variations in temperatures or UV-stress) (Seigler, 1998) or be involved in coloration, odor and even taste in plants (Bennet and Wallsgrove, 1994). The production of those metabolites, often low in terms of dry weight (less than 1%) depends on the developmental and physiological stage of the plant (Rao and Ravishankar, 2002).

Secondary metabolites in pollen could play two major roles in the regulation of the plant-bee interaction. First, they could filter the panel of visitors harvesting floral resources and therefore promote specialization (i.e. oligolectism or even monolectism) (Gosselin et al. 2013). By their potential toxicity, secondary metabolites in natural concentrations could also deter visitors from an excessive intake of pollen (Arnold et al. 2014). Secondary metabolites such as flavonoids are involved in the vivid coloration of gymno- and angiosperms (see Stanley and Linskens, 1974 for review), those molecules being importantly accumulated in pollen grains outer wall (Wiermann and Vieth, 1983). Pollen collected by bees (i.e. bee pollen) has been shown to be a high source of antioxidants, resulting from a significant concentration of polyphenols (flavonoids and phenolic acids) (Marghitas et al. 2009; Nagai et al. 2005). Many studies have thus described the antioxidant effects of those molecules (see point 1.4 for a detailed focus on antioxidants) (Graikou et al. 2011; Kroyer and Hegedus, 2001; Morais et al. 2011). Many other nutrients, precious for health, were reported in bee pollen such as amino acids, vitamins, macro and micro elements (Feas et al. 2012). However, literature on the non-nutritive metabolites in pollen is scarce, and more studies are needed to fully understand their roles.

For nectar, whose role is to reward floral visitors, presence of potentially repellent or toxic secondary compounds seems paradoxical at first view (Detzel and Wink, 1993). Defense compounds in nectar could enhance pollinator service by protecting the flower or prevent an excessive harvest from its floral resources (Adler, 2001).
Among non-nutritive metabolites are reported:

- **Proteins** – while proteins occur at high concentrations in pollen and play a key nutritive role, their presence in nectar seems anecdotic. Even though they are likely to be non-nutritive, the literature is still scarce on the topic. No pollinator attractant or repellent role of proteins has ever been reported in floral nectar, their presence being only linked with a protection against microorganisms and an impact on carbohydrates metabolization. Examples of nectar proteins are nectarins, involved in the nectar redox cycle (Carter and Thornburg, 2004) or invertase, an enzyme allowing the transformation of sucrose into glucose and fructose (e.g. Heil et al. 2015).

- **Protein and non-protein amino acids** - Beyond the 20 amino acids involved in building proteins, more than 250 non-protein amino acids (NPAAs) are found in plants, being involved in the interaction with microorganisms, herbivores and other plants (Vranova et al. 2011). Many NPAAs are known to accumulate in seeds, serving as deterrents to insect feeding (Swain, 1977). Those compounds could potentially present various properties on interacting animals: a direct impact on animal nervous system (Breer and Heilgenberg, 1985), an effect on phagostimulation (Mitchell and Harrison, 1984) or a positive impact on the muscular system (Whitton et al. 1987).

- **Other non-nutritive compounds** - Other potentially defensive compounds are reported in floral nectar, influencing the interaction with many kinds of floral visitors like pollinators (e.g. Hagler and Buchmann, 1993), nectar thieves (eg. Stephenson, 1981; Adler and Irwin, 200), and microorganisms (Thornburg et al., 2003). Alkaloids in nectar have been reported to increase with leaf herbivory, highlighting the dynamism in nectar secondary metabolites profile with the environment (Adler et al. 2006). The same molecules, generally deterring pollinators such as bumblebees (Gegear et al. 2007), are also reported to reduce their parasitism (Manson et al. 2010; Richardson et al. 2015; Baracchi et al. 2015). Many plants also contain phenolics that could play antimicrobial roles and could prevent harvest from robbers, even if Haber et al. (1981) showed that phenolics may not be a decisive barrier against robbing.
1.4. A case of study for secondary metabolites: antioxidants

1.4.1. Generalities

Molecular oxygen reduction to water is a normal metabolic pathway in aerobic cells. Yet, the sequential electron transfer generates free reactive oxygen species (ROS) able to damage carbohydrates, proteins and even DNA, leading to an oxidative stress. Superoxide radicals (O$_2^-$), hydroxyl radicals (OH•), hydrogen peroxide (H$_2$O$_2$) form the main ROS cells must deal with (Simic et al. 1989). Defense mechanisms against those dangerous compounds are provided by a great panel of molecules called antioxidants (Halliwell, 1992), mainly represented by carotenoids, ascorbic acid, tocopherols and flavonoids, and reported in humans to protect against several chronic diseases (Pryor, 1991; Lai et al. 2001).

Among antioxidants, attention is here drawn on flavonoids, natural organic chemicals consisting mainly of flavanones, flavones, flavonols, catechins and anthocyanidins (Herrmann, 1988). Flavonoid is thus a collective noun, used for plant pigments mostly derived from benzo-gamma-pyrone, and synonym with chromone (Hassig et al. 1999; Harborne 1967). Flavonoids are all based on a fifteen-carbon skeleton structured in two benzene rings linked by a heterocyclic pyran ring (Fig. 2). Variations within classes of flavonoids are based on the level of oxidation of the C ring (the heterocyclic pyran ring) and its pattern of substitution, while molecules within a class differ in terms of pattern of substitution of the A and B cycles (Middleton, 1998; Kumar and Pandey, 2013).

Figure 2. Basic flavonoid structure depicting the fifteen-carbon skeleton made of two benzene rings (A and B) linked by a pyran ring (C). From Kumar and Pandey (2013)
In plants, strong correlations have been highlighted between total antioxidant activities (TAA) and total phenolic content (TPC), the latter including flavonoids (Ramaiya et al. 2013; Brighente, 2007) acting as free radical scavengers, metal ions chelators or inhibitors of enzymes involved in free-radical generation (Benavente-Garcia et al. 1997; Acker et al. 1996). Flavonoid activities are structure-dependent and have been reported in humans to play anti-inflammatory, antithrombotic, vasodilatory, antibacterial or antiviral roles (Cook and Samman, 1996).

1.4.2. Roles of flavonoids in plants

With carotenoids and betalains, flavonoids are the main chemicals responsible for the color of plants (Forkmann, 1991) and therefore contribute to their communication with the surrounding environment (Brouillard and Cheminat, 1988). Since flavonoids are ubiquitous in green plants, it has been suggested that they could play a role in photosynthesis (Mukohata et al. 1978) even if no direct involvement in this process has been found. Flavonoids are mainly located in chloroplasts (ROS generation centers) and in the nucleus of mesophyll cells. Their biosynthesis, above all due to oxidative stress, enables UV-B and UV-A absorption (UV being considered as energetic solar wavelengths), inhibits the generation of reactive oxygen species and allows their quenching (Agati et al. 2012). When ROS detoxifying enzymes activities decrease in the chloroplast (due to environmental limitations, e.g. a CO2 assimilation limitation), ROS scavenging flavonoids biosynthesis is upregulated (Ferdinando et al. 2012). In the nanomolar range, flavonoids may also regulate plants growth by being involved in auxin movements (i.e. creating auxin gradients) and catabolism (Taylor and Grotewold, 2005). Moreover, evidence of nuclear location of flavonoids may suggest their role as transcriptional regulators, regulating the activity of proteins involved in cell growth (Saslowsky et al. 2015).

1.4.3. Impacts of flavonoids on insects

Insects have been reported to utilize flavonoids to increase their fitness. For instance, it has been suggested that the accumulation of flavonoids in the wings of butterflies can be used in visual communication, females sequestering more flavonoids being more attractive to
males (Burghardt et al. 2000, 2001). However, those insects have also been shown to be sensitive to some flavonoids contained in leaf resin, the latter being reported as anti-herbivory and protector against UV light (Lincoln, 1985; Lincoln and Walla, 1986). Larvae fed on some flavonoids have also shown reductions in enzymatic activities, some of those compounds being even suggested as usable in insect control programs (Su et al. 2018). The same flavonoids can be reported as both feeding-stimulant and deterrent, depending on the tested concentration (Blaney and Simmonds, 1983). Electrophysiological studies on caterpillars have also shown no impact of the flavonoid rutin on their taste sensilla (Van Drongelen, 1979), the latter known to elicit behavioral responses such as food acceptance or rejection (Schoonhoven and van Loon, 2002). A similar study on the diamondback moth also showed that no neuron from the larvae responded to flavonoids from its host plant (van Loon et al. 2002). Therefore, even though the behavior or fitness of insects can be modulated by flavonoids such as quercetin-derived compounds, the physiological pathway used to perceive them is not known.

1.4.4. Effects of polyphenols on parasitism

Impacts of polyphenols on parasites have been widely reported in literature. Anti-protozoan activity from flavonoids were observed in vitro (Ambrozin et al. 2004) and in vivo (Andrade-Neto et al. 2004). Catechins, the major flavonoid constituents of green tea (Camellia sinensis) were reported to present an inhibitory effect on Plasmodium falciparum growth in vitro (Sannella et al. 2007). Since catechins were reported as potential inhibitors of mammalian facilitative glucose transporter 1 (Naftalin et al. 2003), Slavic et al. (2009) hypothesized this inhibition could explain the inhibitory effects of those molecules on Plasmodium falciparum. They showed effects of catechins on phyllogenetically distant facilitative hexose transporters but could not find hexose transporters were linked with the growth reduction of the malarial agent. Flavonoids reported as antimalarial compounds are reviewed in Kaur et al. (2009).

Several other antiplasmodial activities of polyphenols (and in particular flavonoids) have been reported, for instance with exiguaflavanones from Artemisia indica (Chanphen, 1998), as well as with acacetin, 7-methoxyacacetin and genkwanin, three flavonoids isolated from
Artemisia afra (Kraft et al. 2003). Trypanocidal activity of catechins from C. sinensis was also reported on two different developmental stages on Trypanosoma cruzi, the causative agent of Chagas’ disease (Paveto et al. 2004). The latter study (i) highlights the low concentrations required for anti-parasitism effects of catechins and (ii) suggests catechins can act intracellularly. Some hydrolysable tanins have also been reported to prevent the Trypanosomatidae Leishmania donovani amastigotes from surviving within cells (Kiderlen et al. 2001). Quercetin, a flavonoid known to be involved in the inhibition of the biosynthesis of hsp proteins (hsp 90, hsp70, hsp27) suppressed the induction of the Toxoplasma gondii bradyzoite’s development phase (Weiss et al. 1998).

The mode of action of those compounds has not been fully elucidated yet. It was hypothesized that flavonoids could alter in a dose-dependent manner the adherence between host cells and protozoans, as observed for Plasmodium falciparum (Dormeyer et al. 2006), Sarcocystis neurona, Cryptosporidium parvum and Neospora caninum (Gargala et al. 2005). They could also interfere with signaling pathways involving MAP kinase and PKC as observed in T. gondii (Robert-Gangneux et al. 2000). Indeed, Braga and de Souza (2006) observed in Trypanosoma epimastigotes abnormal chromatin condensation, changes in the membrane structure of their flagella, formation of autophagosomes and incomplete cell divisions.

Flavonoids could also present topoisomerase inhibition activity, as shown by Das et al. (2006) in Leishmania donovani, inhibiting the cell cycle and inducing apoptosis. Protozoan metabolism could also be directly affected, with a flavonoid impact on fatty acid metabolism enzymes (Tasdemir et al. 2006) or on enzymes involved in the energetic metabolism of the parasite (Paveto et al. 2004; Dominguez et al. 2005). Leishmania mitochondrial metabolism was found to be altered by flavonoids, blocking the development of the parasite (Kayser et al. 2002).
2. Invasive plant species

2.1. Generalities

According to Richardson et al. (2000), invasive plants can be defined as naturalized plants producing reproductive offspring at high distances from parent plants, and therefore presenting the potential to spread rapidly over an important area. Many invasive species are characterized by a high attractiveness for native generalist insects (Stout et al. 2006), generally presenting enhanced visual attractants and interesting floral rewards (Rejmánek, 2000). The latter particularities could potentially sort exotic invasive plants as pollination network perturbators, impacting both flora and fauna (e.g. Ghazoul, 2002; Stout and Morales, 2009). Their impacts on native plant species are controversial, sometimes negative by decreasing both visitation rate and reproductive success (Brown and Mitchell, 2001), positive with a facilitative effect for native plants (e.g. Moragues and Traveset, 2005; Lopezaraiza-Mikel et al. 2007) or neutral (Thijs et al. 2012). The effect of invasive plants on fauna could involve a decrease in native specialist pollinators, by facilitating the perpetuation of generalist ones and decreasing floral biodiversity (Cox and Elmqvist, 2000; Traveset and Richardson, 2006). Many invasive plant species take their origin from horticulture, as it is the case in Belgium for Buddleja davidii, Impatiens glandulifera, Fallopia bohemica and F. japonica, Heracleum mantegazzianum, Senecio inaequidens, Solidago gigantea or Rhododendron ponticum (Saad et al. 2009).

2.2. The case of Impatiens glandulifera

Impatiens glandulifera (Fig. 3) is a Himalayan Balsaminaceae introduced in Europe for ornamental purposes during the first half of the 19th century. Since its first record in England as a naturalized alien (Beerling and Perrins, 1993), it invaded a substantial proportion of European countries (Grime et al. 1988) covering above all wetland habitat such as river-sides, widely known to be prone to plant invasions (Planty-Tabacchi et al. 1996). Its fast invasion is allowed by the production of a high number of seeds, ejected up to 5 m from the parent (Könies and Glavac, 1979; Fitch 1976). While generally considered as highly competitive
against native flora (Hulme and Bremner, 2006), studies have shown its presence does not represent a threat to plant diversity in invaded zones (e.g. Hejda and Pyšek, 2006). Nonetheless, its high nectar productivity is highly attractive to native pollinators (Chittka and Schürkens, 2001) such as bumblebees, able to induce a maximum seed set after a single visit (Nienhuis et al. 2009). In invaded areas, the abundance and proximity of *Impatiens glandulifera* to sympatric native species seems to increase their bumblebee visitation rates (Cawoy et al. 2012). The impacts of the presence of this alien species is thus disputed, and further information on its chemistry could help assessing the reasons behind its invasive success in our western countries.

![Figure 3. The Himalayan balsam (*Impatiens glandulifera*) with (a) details of the flowers (photo: Hornbeam Arts) and (b) an example of invaded site (photo by the Landcashire invasive species project). No scales provided with the original pictures.](image)

*Impatiens* species are reported to contain elevated levels of polyphenols, as shown in different vegetative parts of *Impatiens balsamina* (Clevenger, 1958; Fukumoto et al. 1996; Lei et al. 2010) or *Impatiens bicolor* (Hasan and Tahir, 2005). Szewczyk et al. (2016) identified and explored some characteristics of the polyphenols from 6 *Impatiens* species, namely *I. balfourii*, *I. balsamina*, *I. glandulifera*, *I. noli-tangere*, *I. parviflora* and *I. walleriana*. In the latter study, aerial parts from *Impatiens glandulifera* were investigated, and presented a particularly high free radical scavenging activity.
II. Background and objectives

Areas invaded by alien plants are a center of debates in the field of conservation biologists. For bees, and bumblebees in particular, reduction in the diversity of floral resources has been pointed as a factor of decline (e.g. Goulson et al. 2005). Moreover, invaded sites could be expected to present a more restricted panel of nutrients, and therefore to negatively impact bee health.

The present study aims to combine to a previous study led by members of Laboratory of Zoology of the University of Mons. In the latter, the authors expected 5 sites invaded by *Impatiens glandulifera* to be less suitable for the common bumblebee *Bombus pascuorum* in terms of habitat quality. They compared the parasite prevalence of specimens caught in invaded sites and non-invaded sites, expecting organisms from invaded sites to present a higher parasite prevalence. However, the results were contradictory with the original hypothesis, parasite prevalence being higher in non-invaded sites (Fig. 4).

![Figure 4](image.png)

*Figure 4. Prevalence of *Apicystis bombi*, *Crithidia bombi*, *Nosema bombi*, *Nosema ceranae* and *Nosema* sp. in *Bombus pascuorum* for the *Impatiens glandulifera* invaded sites and the non-invaded ones. Parasite prevalences differing significantly from each other, regardless of invasive plant occurrence, are marked with different letters (A, B and C). For each parasite, comparisons between invaded and non-invaded sites were also performed (n.s., non-significant difference; *, *p* ≤ 0.05; **, *p* < 0.01) (Vanderplanck et al., unpublished data).*
No impact of *Impatiens glandulifera* was detected on the genetic diversity of the *Bombus pascuorum* communities in invaded and non-invaded sites, and therefore population genetics in itself could not explain this difference in parasite prevalence. Three hypotheses were thus conceived to explain these significant differences in parasite loads:

1. The abundance of parasite-sharing bees (e.g. mainly *Apis mellifera*) may be lower in balsam-invaded stations. Fewer *Apis mellifera* could explain a lower parasite transmission, explaining that *Bombus pascuorum* workers would be less infested.

2. An alternative hypothesis is a potential pharmacological impact of an original chemistry of the floral resources (e.g. flavonoids) of *Impatiens glandulifera* on bumblebee health. Since the Himalayan balsam is already known to contain flavonoids (Vieira et al. 2016; Szewczyk et al. 2016) and that flavonoids present multiple potential effects on parasites (see introduction above), we make the hypothesis that flavonoids contained in *Impatiens* floral resources could explain bumblebee parasite prevalence.

3. The last hypothesis is a better habitat quality of the chosen sites before the blooming of *Impatiens glandulifera*, with wild plants providing more pollen and/or better-quality pollen. Larvae fed on this pollen would have grown as healthier adults, explaining the observed differentiation in parasite prevalence.

The aim of this master’s thesis is to test those hypotheses to understand what could explain a lower parasite prevalence in *Bombus pascuorum* workers from the invaded sites. To test the first hypothesis, the visitor community of *Impatiens glandulifera* has been studied in the same sites studied previously, and the relative abundance of the visitors has been compared to non-invaded sites. The panel of floral visitors was characterized by catching sessions and palynological analyses of the pollen loads of *Bombus pascuorum* workers have allowed to understand the importance of this plant in the latter’s foraging activity and diet.

The second hypothesis was tested by analyzing the polyphenols in the floral resources of the Himalayan balsam, those compounds already reported for their antiparasitic effects (see introduction). Finally, wing morphology of the workers of *Bombus pascuorum* was used as a
proxy of habitat quality of their pre-imago stages, and thus as an estimator of the floral assemblage before the blooming of Impatiens glandulifera.
III. Material and methods

1. Plant-visitor associations

1.1. Plant visitors

To test the first hypothesis (i.e. that a lower parasite prevalence could be explained by a lower abundance of parasite-sharing bees in invaded sites), catching sessions were organized and floral visitor communities were compared. The ten sites of approximately 5m x 30m had previously been selected in 6 localities near Mons, Belgium: Dour, Havré, la Louvière, Mons, Quévy and Tertre (Fig. 5). Five of them included *Impatiens glandulifera* at the same density (85 ± 38 flowers/m², $F_{4,15} = 2.51$, $p = 0.086$) while the five others did not include the alien species. As bumblebees share pathogens with honeybees (Graystock *et al.* 2013), a survey of the local beekeepers had been conducted to avoid sites with potential high densities of hives (CEIAM, *Centre d’étude et d’information apicole de Mons*).

Each week for 4 weeks (July-August 2017), catching sessions were organized in the 10 same stations studied previously, 5 of them being invaded by *Impatiens glandulifera* and the 5 others not. For each session, all visitors in contact with the flowers of the plants were caught by net in 2m x 2m squares for 20 minutes, for a total of more than 13 hours of catching. In invaded sites, 4m² squares always contained *Impatiens glandulifera*. In the non-invaded sites, 4m² floristically representative of the ecosystems were chosen. All specimens were killed by freezing at -80°C for an optimal preservation of the pollen loads (see below).

Collected visitors were identified to the genus or species level. Bumblebees were identified using the West-Palaearctic Bumblebees Identification Key (Rasmont *et al.* unpublished data). Due to the degree of uncertainty of a visual species-level identification in the *Bombus terrestris* group (i.e. including *B. cryptarum*, *B. lucorum*, *B. magnus* and *B. terrestris*, see Carolan *et al.* 2012), the operational taxonomic unit (OTU) *Bombus terrestris s.l.* was used in the results. Other bee taxa were identified using identification keys from the BELBEES project (Pauly, 2015a, b). Reference specimens from the Laboratory of Zoology of the
University of Mons were used to perform identifications when species-level identification keys were not available.

Figure 5. **Above**: map of Belgium with the studied sites highlighted. **Below**: zoom on the studied sites (Mons region and close surroundings) with, from left to right, (1) Dour 2, 50.456083°N-3.70338°E; (2) Dour 1, 50.450126°N-3.710761°E; (3) Tertre 1, 50.456493°N-3.812996°E; (4) Tertre 2, 50.473853°N-3.826917°E; (5) Mons 2, 50.452599°N-3.904213°E; (6) Mons 1, 50.470986°N-3.947879°E; (7) Quévy 50.373565°N-3.966569°E; (8) Havré 1 50.468623°N-4.047743°E; (9) Havré 2 50.472993°N-4.063042°E; (10) La Louvière, 50.492218°N-4.181167°E. On both maps, invaded sites are depicted by pink circles and non-invaded ones by orange triangles.
1.2 Palynology

In order to check if *Bombus pascuorum* workers eat pollen from *Impatiens glandulifera*, a total of 101 pollen loads (i.e. corbicula contents, Fig. 6) from *B. pascuorum* workers caught in invaded sites in 2014, 2015 and 2017 were lyophilized, crushed and acetylated to assess the proportion of the invasive plant in their diet. Pollen samples were individually mixed with glacial acetic acid for 30 min in eppendorfs, then centrifuged (10 min, 3500 rpm). Supernatant was removed and substituted by an acetylation mixture (acetic anhyd: sulfuric acid, 9:1). After vigorous centrifugation, the solution was incubated 3 min in a 100°C bath. The samples were centrifuged (10 min, 3500 rpm), supernatant was removed, and the same process was repeated with distilled water until supernatant became transparent and colorless. Water was finally removed and acetylated pollen grains were mounted on microscope slides with melted glycerinated gelatin for palynology. After palynology, processed pollen was cleared of its pollen loads, allowing a better visualization of the exine details.

Figure 6. Pollen loads are the corbicula contents of bees, as shown here with (a) a pollen load on the metatibia of a *Bombus pascuorum* worker (Photo Mark Burnett, scale not provided with the original picture) and (b) trap to allow easy access to the pollen loads without hurting the bumblebee in the field, with a *Bombus pascuorum* worker inside (photo G. Ghisbain). Red circles depict pollen loads in both pictures.
The proportion of *Impatiens glandulifera* on the slides was assessed with an optical microscope (400x magnification, Fig. 7). Identification was unambiguously performed using pure acetolysed pollen from *I. glandulifera* as a reference slide. The field of vision of the microscope was first placed at the corner of a slide, and the proportion of *I. glandulifera* pollen grains was recorded. The field of vision was then shifted horizontally to once its width (in order not to count a same pollen grain twice), before another record. Once at the end of the slide, the field of vision was shifted vertically to twice its width, followed by horizontal progression as explained. Counting was stopped at 300 pollen grains, and the proportion of *I. glandulifera* in the samples was calculated as the number of its pollen grains divided by 300 (inspired from Somme et al. 2015). A total of 101 pollen loads were analyzed to acquire sufficient sampling. All years were pooled because of the heterogeneity in the availability of pollen loads samples regarding the year.

Figure 7. Microscopic view of a pollen load after treatment by acetylosis. Morphology of the pollen exine helps sorting grains by morphotypes or even species. Photo by Ghisbain G.
2. Analytical chemistry on floral resources from *I. glandulifera*

In order to test the second hypothesis (i.e. secondary metabolites from the floral resources could explain a lower parasite prevalence by a medicinal effect), pollen and nectar were collected in each invaded site from *Impatiens glandulifera* flowers previously bagged with nets. Nets were placed on flower buds to avoid any contact and consumption of the floral material by visitors before sampling. Pollen and nectar samples (*n* = 5) were then stored at -80°C. The polyphenol content was assessed from an accurately weighed amount of lyophilized pollen (i.e. 5-10 mg) placed in a 1.5 mL microcentrifuge tube. Polyphenols were extracted in presence of aqueous methanol (methanol:water 50:50) using an ultrasonic bath during 15 minutes. Following centrifugation at 13000 rpm for 2 min, the clear supernatant was transferred to a vial. This extraction procedure was repeated four times per sample (i.e. 4 extractions with 200 µL methanol:water 50:50, supplemented with the same solvent to reach exactly 1 mL, adapted from Szewczyk *et al.* 2016). The whole protocol for the extraction of polyphenols from pollen is depicted in Fig. 8.

Extracts were then filtered and transferred in UHPLC vials prior to injection of 10 µL in the analytical system. All analyses were performed at the Medical University of Warsaw (Poland) in collaboration with the Dr. S. Granica. Nectar was simply diluted 200 and 20 times with the extraction solvent prior to filtration and injection of 10 µL in the analytical system. As none compound was detected in the diluted nectar the raw nectar was also injected.

Polyphenols were profiled by UHPLC-DAD-MS. An Ultimate 3000 series system (Dionex. Idstein. Germany) equipped with a diode array detector coupled with an Amazon SL ion trap mass spectrometer was used (Bruker, Bremen, Germany). The separation was performed on a Kinetex XB-C18 column (150 mm x 3.0 mm x 2.6µm, Phenomenex, USA). Temperature was maintained at 25°C. The flow rate was 0.4 mL/min and a gradient elution with mixtures of formic acid 0.1% in water (phase A) and in acetonitrile (phase B) was used as follows 5-26% B 0-60 min. 26-50% B 60-70 min and to 95% 70-75 min. The parameters for ESI source of ion trap were: nebulizer pressure 40 psi; dry gas flow 9 l/min; dry temperature 300 ºC; and capillary voltage 4.5 kV. Analysis was carried out using a scan range from *m/z* = 70 to *m/z* =
The MS spectra of detected compounds were recorded in the positive ion mode. Standards of ampelopsin and isoquercetin (both at 1mg/mL in DMSO) were prepared using purchased chemical standards all >95% HPLC purity.

**Figure 8. Protocol for the extraction of polyphenols from pollen, adapted from Szewczyk et al. 2016.**

Figure by G. Ghisbain, 2017
3. Geometric morphometrics on wings from *B. pascuorum* workers

To test if the observed parasite prevalence dissimilarity could be associated by a difference in habitat quality before the blooming of *Impatiens glandulifera* (and therefore a difference in pre-imago nutrition of the collected workers), the wing morphology of *Bombus pascuorum* workers in all stations was compared. It is now assumed that body size depends on various factors such as resource suitability (Roulston and Cane 2000), is related to fitness components (Chown and Gaston, 2010) and decreases in response to diverse stresses (Gérard *et al.* sub.). Wing morphology can therefore be considered as a proxy of local bumblebee population health or development prior to the blooming period of the Himalayan balsam.

Right forewings from *Bombus pascuorum* workers were cut off at the level of the tegulae (i.e. base of the wing) to assess wing size and shape differences between bumblebees from invaded sites and non-invaded sites. Wings from 2014, 2015 and 2017 were sampled to acquire a representative dataset for statistical analyses. 240 pairs of wings from 2014 and 2015 (120 per year, 60 from invaded stations, 60 for non-invaded ones) and 105 from 2017 (55 from invaded stations, 50 from non-invaded ones) were sampled. Wings were individually isolated, placed on graph paper, hold tight between microscope slides and photographed using an Olympus SZH10 microscope coupled with a Nikon D200 camera. Wings were input to tps-UTILS 1.56 (Rohlf, 2013a). Two dimensional Cartesian coordinates of 18 landmarks (Table 1 in annex) (Fig. 9) were used to capture wing shapes. This process, called digitization was performed using tps-DIG v2.17 (Rohlf, 2013b). Parallelly, the pictures were scaled.

![Figure 9. Wing of a bumblebee with the 18 landmarks used in this study (Gérard *et al.* 2015)](image-url)
The 345 bidimensional Cartesian landmark configurations were scaled, translated and rotated against the consensus configuration using the Generalized Least Squares (GLS) Procrustes superimposition method. This process allowed to (i) separate size and shape components of the wing and (ii) to remove all non-shape differences. Practically, the GLS method minimalizes differences between homologous landmark configurations using Procrustes distance as a criterion (Fig. 10). The Procrustes distance corresponds to the sum of the squared distances between corresponding landmarks. This method thus calculates the centroid of each landmark configuration, performs a superimposition according to the centroids and minimalizes the Procrustes distances between homologous landmarks (Zelditch et al. 2004). In the end, centroid size was calculated as a proxy of the size of the individuals.

\[ d_{xy} = \sqrt{\sum_{i,j}^{k,p} (X_{i,j} - Y_{i,j})^2} \]

Figure 10. Procrustes distance formula (from Zelditch et al. 2004)

4. Data analyses

4.1. Plant-visitor associations

Differences in pollinator communities were assessed using perMANOVA (Euclidean distance matrix, 999 permutations, “adonis” command) after testing for multivariate homogeneity (“betadisper” command) (R-package vegan, Oksanen, 2017). Indicator compound analyses were also performed to identify pollinators that were indicative of a site-type (“indval” command) (R-package labdsv, Roberts, 2016). All these analyses were conducted in R version 3.4.0 (R Core Team, 2017). Data were expressed as arcsin-transformed proportions.
4.2. Wing morphometry

4.2.1. Wing size

After checking for residuals normality (Shapiro-Wilk test) and data homoscedasticity (Bartlett test), data were normalized using the rtranform function (R-package “GenABEL”) and a two-way nested analysis of variance (ANOVA) was used to compare centroid sizes (CS) of wings between both site-types taking into account the site effect. All these analyses were conducted in R version 3.4.0 (R Core Team. 2017).

4.2.2. Wing shape

Principal component analysis (PCA) was performed to investigate a potential shape variation amongst both groups, using the geomorph function “PlotTangentSpace” in R. PerMANOVAs (vegan function “adonis”, permutations = 999, Euclidian distances) were then applied to the principal components calculated for each individual (after multivariate homogeneity was tested) to explore if the type of site on which bumblebees were collected could impact wing shape.
IV. Results

1. Floral visitors in invaded and non-invaded sites

1.1. Comparison of visitor communities

Results show a clear impact of the balsam on the floral visitor communities in the studied sites (Fig.11 and 12). According to the perMANOVA, invaded and non-invaded sites showed significantly different visitor communities ($F_{1,9} = 5.64, p = 0.014$). *Bombus pascuorum* was the main floral visitor in all sites, with an average prevalence of $82.5 \pm 6.2\%$ (mean $\pm$ SEM) in invaded sites and $46.4 \pm 6.3\%$ in non-invaded sites, those prevalences presenting a significant difference ($IC = 0.64, p = 0.013$). Another significant difference was observed for the bumblebee species *Bombus lapidarius*, the latter being more abundant in non-invaded sites ($0.6 \pm 0.06\%$) than in invaded sites ($11.2 \pm 3.5\%$) ($IC = 0.95, p = 0.020$). The last collected bumblebee species, *Bombus terrestris s.l.*, did not show any significant prevalence difference regarding site type (invaded sites: $11.9\% \pm 3.6\%$, non-invaded sites: $10.3 \pm 6.4\%$). The honeybee *Apis mellifera* showed a prevalence of $3.5 \pm 2.4\%$ in invaded sites and $9.7 \pm 3\%$ in non-invaded sites, those results not significantly differing regarding site type.

![Visitor profiles in invaded and non-invaded sites](image)

Figure 11. Mean percentages ($\pm$SEM) in visitor profiles for sites invaded by *Impatiens glandulifera* (grey) and non-invaded sites (orange). Each site received the same sampling effort. Difference in visitor profiles was assessed by perMANOVA ($F_{1,9} = 5.64, p = 0.014$).
Figure 12. Proportions of floral visitors in invaded (above) and non-invaded sites (below) depicting more visually the impact of the balsam on the floral visitor communities in the studied sites. Note the importance of *Bombus pascuorum* regardless site-type.
1.2. Palynology on the pollen loads from Bombus pascuorum workers

The average concentration of *Impatiens glandulifera* in the pollen loads (all years pooled, N=101) reached 23.65%. Approximately 70% of them contained from 0 to 25% of *Impatiens glandulifera* while 10.89% contained nearly pure pollen from this plant (from 75 to 100%). Whereas nearly 20% did not contain pollen from the invasive plant, 6 pollen loads (5.9%) contained more than 99% of *Impatiens glandulifera* (Fig. 13).

Figure 13. Percentages of *Impatiens glandulifera* in the pollen loads collected in 2014, 2015 and 2017 (N=101). Green color represents pollen loads containing from 0 to 25% of Himalayan balsam in the sample, red from 25 to 50%, yellow from 50 to 75% and orange from 75 to 100%.
2. Polyphenolic composition of pollen and nectar from *Impatiens glandulifera*

Whereas no compound was detected in the 5 nectar samples, five polyphenols were identified and quantified from pollen, the flavonoid ampelopsin being the major one (13.81 ± 1.26 mg/g). A representative polyphenolic profile detected in pollen is presented in Fig. 14. Quantification results of polyphenols from the pollen of the five invaded stations is shown in table 1.

![Figure 14. Representative chromatogram of a pollen sample recorded at 254 nm. 1 = unidentified compound, 2 = ampelopsin, 3 = ampelopsin isomer, 4 = myricetin O-rhamno-O-hexoside t, 5 = quercetin O-rhamno-O-dihexoside t and 6 = kaempferol O-rhamno-O-dihexoside t](image-url)
Table 1. MS and UV-Vis data of compounds detected in pollen samples and their quantification (N=5)

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<thead>
<tr>
<th>Compound name</th>
<th>retention time [min]</th>
<th>UV [nm]</th>
<th>[M+H]+ m/z</th>
<th>MS² ions</th>
<th>MS³ ions</th>
<th>Mean concentration (mg/g) ± SD</th>
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<tr>
<td>1 unidentified compound</td>
<td>19.6</td>
<td>212, 254</td>
<td>502</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>2 ampelopsin †</td>
<td>21.2</td>
<td>205, 225sh, 291</td>
<td>321</td>
<td>303, 275b, 247, 195, 153</td>
<td>-</td>
<td>13.81 ± 1.26</td>
</tr>
<tr>
<td>3 ampelopsin isomer †</td>
<td>26.0</td>
<td>211, 225sh, 294</td>
<td>321</td>
<td>303, 275, 195, 153b</td>
<td>-</td>
<td>0.99 ± 0.14</td>
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<tr>
<td>4 myricetin O-rhamno-O-hexoside †</td>
<td>28.1</td>
<td>254, 264, 355</td>
<td>627</td>
<td>481, 319b</td>
<td>319b</td>
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<tr>
<td>5 quercetin O-rhamno-O-dihexoside †</td>
<td>32.7</td>
<td>253, 263sh, 355</td>
<td>773</td>
<td>686, 627, 461, 303b</td>
<td>465b, 447, 411, 369, 303</td>
<td>0.25 ± 0.13</td>
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<td>6 kaempferol O-rhamno-O-dihexoside †</td>
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<td>265, 349</td>
<td>757</td>
<td>611, 449, 287b</td>
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<td>2.82 ± 0.77</td>
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</table>

* † tentative assignment, s - comparisons with chemical standard were made, b - base peak (the most abundant ion in recorded spectrum), in bold - ions subjected to MS³ fragmentation. Compounds 2 and 3 were quantified in ampelopsin equivalents, compounds 4 to 6 in isoquercetin equivalents.
3. Morphological analyses of Bombus pascuorum wings

Wing size - Although a significant centroid size difference was detected regarding site-type ($F_{1,678} = 79.78, p < 0.001$), non-invaded sites were not more similar to one another than to invaded sites (two-way nested ANOVA, type:site effect, $F_{8,678} = 10.16, p < 0.001$). For instance, the invaded site Dour 1 did not differ significantly from the non-invaded Havré 2 ($p=1$), while both invaded sites Dour 1 and Quévy significantly differed ($p < 0.001$).

Wing shape – The global overlap of wing configurations from both invaded and non-invaded sites regarding the two first principal component axes of the Principal Component Analysis (PCA) does not suggest a clear differentiation regarding site-type (Fig. 15). Moreover, while the results of the perMANOVA suggest a significant difference between the groups ($p =0.006$), the very low value of the $R^2$ (0.0076) tends to show that alternative mechanisms structured the wing shape differentiation. Therefore, habitat quality is probably not the major factor of the wing shape variation in the tested individuals.

![Figure 15. Ordination of the two groups of Bombus pascuorum wings (red: from non-invaded and blue: from invaded sites) along the first two axes of the principal component analysis. The overlap of wing configurations for both invaded and non-invaded sites tends to show that wing shape is not associated with site type.](#)
V. Discussion

1. Impacts of Impatiens glandulifera on the floral visitor communities

Site-type differences were observed both at the community level and at the species level. Bombus pascuorum and Bombus lapidarius showed opposite associations, the former with invaded sites and the latter with sites where the Balsam was absent. Moreover, the palynological analyses of pollen loads from Bombus pascuorum workers revealed a collection of the Balsam’s pollen for dietetic purposes.

The site-type significant difference in bee communities seems to corroborate with many studies highlighting a competitive effect of alien plants on native ones for flower visitors (e.g. Chittka and Schürkens, 2001; Ghazoul, 2002 and Larson et al. 2006). Moreover, Lopezaraiza-Mikel et al. (2007) showed that generalist insects were likely to provide a pathway of integration for Impatiens glandulifera, recording Apis mellifera and Bombus pascuorum as the main visitors of the alien. Even though Apis mellifera was not a main pollinator in this study, we cannot conclude on its usual prevalence in the visitor community because the sites were deliberately chosen far from honeybee hives.

Bombus pascuorum is the most widespread and most abundant European bumblebee, even in massively human-transformed landscapes (Goulson et al. 2008; reviewed at the continental scale in Rasmont et al. 2015). Since it is a polylectic forager (Leclerq, 1960; Teräis, 1985), it is not surprising to find it in both invaded and non-invaded sites. Moreover, its abundance on the invasive species Impatiens glandulifera had already been reported in other studies (e.g. Lopezaraiza-Mikel et al. 2007; Nienhuis et al. 2009) where it is described as the principal bumblebee species visiting this plant. Nienhuis and Stout (2009) highlighted the high efficiency of B. pascuorum as a pollinator of I. glandulifera due to (i) its high visitation frequency, (ii) its morphological fit with the flowers and therefore (iii) its ability to remove important pollen quantities from the anthers, inducing a maximum seed set after one single visit.
The attractiveness of *Impatiens glandulifera* could be explained by a massive production of nectar, and a high rate of sugar production included in the latter (Chittka and Schürkens, 2001). This invasive plant species has therefore been regarded as competing for or facilitating pollinator visitation to the native flora (Chittka and Schürkens, 2001; Lopezaraiza-Mikel *et al.* 2007). In contrast, *Impatiens glandulifera* may benefit native pollinators by supplying important quantities of floral resources (Showler, 1989; Starý and Tkalcu, 1998). The present study also highlights the fact that *I. glandulifera* may benefit some generalist pollinator species like *B. pascuorum*, increasing its relative abundance (i.e. proportion) in invaded sites, while other species like *B. lapidarius* do not seem to take advantage of the presence of this plant in the studied sites.

From the palynological point of view, even though most pollen load samples from *Bombus pascuorum* workers contained a low percentage of *Impatiens glandulifera*’s pollen, the average percentage of this species in the pollen loads indicates that pollen from this plant is foraged by the bumblebees and must therefore be given to the larvae. Previous studies reported that *Bombus pascuorum* generally presents a broad diet, at least broader than the other polylectic and abundant bumblebee *B. terrestris* (Carvell *et al.* 2006; Leonhardt and Blüthgen, 2012) even though local and temporal conditions might better explain pollen collection than fixed and specific behavior (Roulston and Goodell, 2011; Somme *et al.* 2015). Moreover, most *Bombus pascuorum* could visit the flowers above all for nectar, the latter being highly attractive to bumblebees in terms of sugar concentration (Chittka and Schürkens, 2001). Behavioral observations in the field also tend to show that most *Bombus pascuorum* consume the nectar deep in the corolla and less commonly manipulate the anthers (per. obs.). While the total amino acid content of *I. glandulifera* content is low (e.g. penultimate of the 25 taxa studied in Roger *et al.* 2016), the sterol content could better explain an attraction for this pollen (more concentrated than 6/18 taxa studied in Roger *et al.* 2016).
2. Bee community and parasite community

Community-level differences regarding site-type, including the absence of significant difference in the abundance *Apis mellifera*, should be considered with regard to the trends in parasite prevalences previously observed in *Bombus pascuorum*. The formerly suggested hypothesis aiming to link the parasite dynamics in *B. pascuorum* with the honeybees might now be directed towards another bee species, *B. lapidarius*, whose significant difference in abundance was detected between invaded and non-invaded sites.

First, the significantly higher prevalence of the bumblebee *Bombus pascuorum* in invaded sites tends to weaken the parasite-sharing bee hypothesis, as a higher parasitism prevalence could be expected where a massive proportion of conspecific organisms share the same resources. Then, previous studies highlighted honeybee-bumblebee parasite transfer (such as *Apicystis bombi*, *Crithidia* spp. and *Nosema* spp.) (Cameron *et al.* 2011; Graystock *et al.* 2013) and Fürst *et al.* (2014) reported honeybees as important vectors of EIDs (Emerging infectious diseases) able to infect wild bumblebees. Overall, many other studies reported that wild pollinators such as *Bombus* spp. were associated with honeybee pathogens in shared areas (e.g. Genersch *et al.* 2006; Evison *et al.* 2012). Here, the similarity in *Apis mellifera* ratios between invaded and non-invaded sites strongly supports the hypothesis whereby the lower parasite prevalence in invaded sites could well be due to *I. glandulifera* and not to the lower abundance of the honeybee.

A significant difference in potentially parasite-sharing bee was however observed for another bee species, *Bombus lapidarius*. The latter is a very common bee species in Europe that had already been observed as a floral visitor of *Impatiens glandulifera* (Starý and Tkalcu, 1998). In this sampling, even though its relative abundance was significantly higher in non-invaded sites, we can consider that its very low frequency regarding site-type seems not likely to explain any important parasite transmission in non-invaded sites at first view. However, *Apicystis bombi* (pers. obs.), *Crithidia bombi* (Ruiz-González *et al.* 2012) and *Nosema bombi* (Larsson, 2007) have already been detected in this species, and the impact of the presence of
B. lapidarius on parasite transmission would still deserve deeper attention.

3. Secondary metabolites in the pollen of Impatiens glandulifera

As predicted based on studies revealing the presence of polyphenolic compounds in the aerial parts and flowers of Impatiens species (Szewczyk et al. 2016; Vieira et al. 2016), the latter compounds were detected in the floral pollen of the alien plant.

From the 5 polyphenols detected and identified in Impatiens glandulifera’s pollen, a particular attention is drawn on ampelopsin. Also known as dihydromyricetin, ampelopsin (C\textsubscript{15}H\textsubscript{12}O\textsubscript{8}, fig. 16) is a well-known polyphenol already reported for its in vivo anti-oxidative stress properties (He et al. 2003; Murakami, Miyakoshi, Araho et al. 2004). This flavonoid may confer multiple pharmacological roles in well-known medicinal herbs such as Ampelopsis grossedentata, from which leaves are used in China (inter alia) to prepare the medicinal Rattan tea (Kou and Chen, 2012).

\begin{figure}[h]
\centering
\includegraphics[width=0.3\textwidth]{ampelopsin_structure.png}
\caption{Structure of the flavonoid ampelopsin (dihydromyricetin)}
\end{figure}

Even though most known effects of this molecule are reported for humans and mice, it is interesting to have an idea of the range of action of this compound. Effects of non-cytotoxic levels of ampelopsin range from anti-inflammatory (Qi, Xin, Guo et al. 2012) to inhibitor of both growth and invasion of various tumors and cancers in vivo and in vitro (Liu et al. 2003; Zheng and Liu, 2003; Zeng et al. 2004; Luo, Zeng and Liu, 2006; Ni et al. 2012). These reports suggest that ampelopsin could exert its effects by modulating diverse signaling pathways at multiple cellular levels, impacting apoptosis, cell cycle arrest, cell growth and metastatic inhibition (reviewed in Kou and Chen, 2012) or even by increasing cellular
antioxidant defense (Kou et al. 2012).

The presence of ampelopsin in all analyzed samples of pure pollen strongly suggests that this flavonoid is ingested by visitors collecting pollen from *Impatiens glandulifera*. Ampelopsin was reported as a food stimulant in *Plagiodera versicolora*, a Chrysomelid beetle species often found on the trees of *Salix sachalinensis* whose leaves contain significative levels of this molecule (Matsumoto, 2000). Even if the effects of ampelopsin are not known on bumblebees, an increase in immunological functions due to its antioxidant and anti-microbial effects could be considered to explain a lower parasite prevalence. Indeed, effects of ampelopsin on microorganisms such as bacteria (Xiong et al. 2000) and fungi (Xiong et al. 2004; Matsumoto and Tahara, 2001) have been reported. For the moment, ampelopsin has not been reported as anti-parasitic, in contrast with other polyphenols that have demonstrated inhibiting properties against the parasite causing malaria, *Plasmodium falciparum*, belonging to the same phylum as *Apicystis bombi* (Slavic et al. 2009).

Curative properties of other secondary metabolites have already been demonstrated. Anabasine and nicotine (both alkaloids), catalpol (an iridoid glycoside) and thymol (a terpenoid) significantly reduced the infection level of *Crithidia bombi* in *Bombus impatiens* by as much as 81% (Richardson et al. 2015). Manson et al. (2010) detected a reduction of the faecal intensity of *Crithidia bombi* seven days after infection when bees were fed continuously with the indol alkaloid gelsemine. However, inoculation of *Crithidia bombi* with gelsemine prior to infection did not show a significant trend towards a reduced infection.

In contrast with the positive impacts of secondary metabolites, bees have been shown to experience negative effects of consuming plant chemicals. Detzel and Wink (1993) demonstrated that some secondary metabolites could be unpleasant and even toxic to *Apis mellifera*, even if no link could be highlighted between food rejection and toxicity. Their data show that bees that are confronted with plants allelochemicals are not especially adapted to the chemical defense of plants. Arnold et al. (2014) showed that fewer and smaller males were produced by microcolonies fed with pollen supplemented with lupanine, an alkaloid naturally found in *Lupinus* spp. pollen, at ecologically-relevant concentrations. In particular, phenolic
and flavonoid compounds have already been reported to drastically impact growth and progeny of an insect (Homoptera, Aphidae) (Todd et al. 1971).

4. Habitat quality before the blooming of Impatiens glandulifera

Analyses both on wing shape and size from collected Bombus pascuorum indicate that habitat quality prior to the blooming of the invader was not a main factor able to explain the previously observed parasite prevalence differentiation. We can therefore assume that resource suitability, abundance and/or floral assemblage was likely to be similar regarding site type.

Indeed, previous studies have reported that various stressors such as secondary compounds (Padro et al. 2014), parasitism (Polak, 1993) and inbreeding (Fredrickson and Hedrick, 2002) can impact morphological traits, including shape and size. Food quantity and protein richness have also been shown to dramatically impact size in a Lasioglossum bee (Hymenoptera, Halictidae) (Roulston and Cane, 2002). Wing shape and size have already been reported as relevant indicators of environmental stresses in a moth (Lepidoptera, Noctuidae) (Hoffmann et al. 2002). Here, larger wings (and therefore larger individuals) would have been expected in environments where food availability was higher, which is not the case here. Size would have also decreased in response to diverse stresses such as toxins, parasites, temperature and inbreeding (Gérard et al. sub.). We therefore consider that all Bombus pascuorum, regardless the site-type, were in similar development conditions before their imago stage. Thus, the difference in parasite prevalence is not likely to be explained by the floral assemblage before the blooming of Impatiens glandulifera.
VI. Conclusion

As an alien plant species, the Himalayan balsam has been the center of debates in conservation biology. This work confirms that the presence of this plant has an impact on the community of floral visitors in invaded sites. While the common carder bee *Bombus pascuorum* has been shown to take advantage of the presence of the alien, the red-tailed bumblebee *Bombus lapidarius* was relatively more abundant in sites that did not include the invader.

This work highlights the fact that pollen from this plant is utilized (~23%), at least by the workers of *Bombus pascuorum*. Besides the impact of the alien in the visiting bee communities of the ecosystems it is included in, the presence of its pollen grains in the pollen loads of *Bombus pascuorum* workers shows that the invader could have been adopted by the species as part of its pollen diet.

Floral assemblage preceding the arrival of the invader is very likely not to explain any strong morphological differentiation regarding site-type. We therefore invalidated the hypothesis suggesting that a significantly better habitat quality before the blooming of the alien was a main factor explaining the difference in parasite communities between the sites.

Chemical analyses have revealed an interesting polyphenolic profile in the pollen of *Impatiens glandulifera*. While other studies had shown that polyphenols were common in the Balsaminaceae family, no previous work had focused on their pollen. The presence of polyphenols being often associated with important antioxidant effects, it is all the more fascinating to see that this pollen is included in the corbicula contents of a bumblebee species.

In the end, this work succeeded to encircle the observed impact of the Himalayan balsam on bumblebee parasitism. The results suggest that a rich polyphenolic profile of the Balsam’s pollen could positively affect the health of the native bumblebee *Bombus pascuorum* in alien-invaded sites. While invasive species are often regarded as ecological disasters, this
work shows that aliens can also bring with them beneficial properties to our native ecosystems.
VII. Perspectives

This work opens a large panel of perspectives, above all in the fields of pollinator and landscape conservation.

First, although pollen chemistry starts to be elucidated for this plant, its direct impact on bumblebee colony development is unknown. Testing the effects of a ~23% Impatiens glandulifera diet on bumblebees and comparing it with controls (i.e. a poorly nutritive diet of pure Taraxacum and a highly nutritive one based on Salix) would help to estimate if this resource could really be considered as beneficial for wild populations. Since the invader’s pollen is not commercially available, growing I. glandulifera in greenhouses including the colonies could be considered. We could then quantify the impacts of this plant on colony development parameters such as mortality, egg spawn, production of larvae and pollen efficiency. Metabolomics would then be a supplementary step to assess if an Impatiens-based diet tends to direct metabolism closer to the one resulting from a Taraxacum-based or Salix-based diet. Looking at the sequestered molecules such as flavonoids would also help to understand how such compounds are metabolized, and at which step of development they are the most important (e.g. pre- or post-imago).

A study depicting the direct effects of the flavonoid ampelopsin on bumblebees is already ongoing at the Laboratory of Zoology of the University of Mons. Preliminary results show that the permanent consumption of Salix pollen supplemented with this molecule at the same concentration than in natural Balsam’s pollen does not affect both pollen and nectar consumption by bumblebee workers, compared to a group fed with non-supplemented pollen (Ghisbain et al., unpublished data). Further analyses will focus on the consequences of ampelopsin ingestion on the profile of primary metabolites in hemolymph and on the panel of bacteria species of the bumblebee’s gut. Those analyses will be performed in collaboration with the University of Gent (Belgium).

Natural and human-induced stressors now act in synergy at the world scale,
permanently modeling and sometimes drastically changing our landscapes. Living in a changing world will therefore require more efforts to better understand what are the real ecosystemic consequences of both arrival of alien populations and adaptation of native ones.
VIII. References


Chittka L., Thomson J.D. and Waser N.M. (1999). Flower constancy, insect psychology, and


Whitehorn P.R., Wallace C. and Vallejo-Marín M. (2017). Neonicotinoid pesticide limits improvement in buzz pollination by bumblebees, *Scientific Reports, 10.1038/s41598-*. 


IX. Appendices

Table 1. Positioning and typology of the 18 landmarks used in the present study, following Owen (2009).

Table 2. Floral visitors recorded in the 5 invaded and 5 non-invaded sites in 2017, expressed as proportions.
Table 1. Positioning and typology of the 18 landmarks used in the present study, following Owen (2009). Definitions correspond to the digitization of a right wing.

<table>
<thead>
<tr>
<th>Landmark number</th>
<th>Position of the landmark on a right wing or a mirror-reflected left wing</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Internal point situated at the extreme right of the marginal cell</td>
<td>Ⅱ</td>
</tr>
<tr>
<td>2</td>
<td>Internal point situated at the extreme left of the marginal cell</td>
<td>Ⅱ</td>
</tr>
<tr>
<td>3</td>
<td>Point situated at the intersection between the Rs vein and the second [abscissa] of the Rs vein.</td>
<td>I</td>
</tr>
<tr>
<td>4</td>
<td>Point situated at the intersection between the Rs vein and the 1rs-m vein</td>
<td>I</td>
</tr>
<tr>
<td>5</td>
<td>Point situated at the intersection between the Rs vein and the 2rs-m vein</td>
<td>I</td>
</tr>
<tr>
<td>6</td>
<td>Point situated at the intersection between the M vein and the 2rs-m vein</td>
<td>I</td>
</tr>
<tr>
<td>7</td>
<td>Point situated at the intersection between the M vein and the 2m-cu vein</td>
<td>I</td>
</tr>
<tr>
<td>8</td>
<td>Point situated at the intersection between the 1rs-m vein and the M vein</td>
<td>I</td>
</tr>
<tr>
<td>9</td>
<td>Point situated at the intersection between the 1m-cu vein and the M vein</td>
<td>I</td>
</tr>
<tr>
<td>10</td>
<td>Point situated at the intersection between the second [abscissa] of the Rs vein with the M+Rs vein</td>
<td>I</td>
</tr>
<tr>
<td>11</td>
<td>Internal point situated at the extreme bottom-left corner of the first submarginal cell</td>
<td>Ⅱ</td>
</tr>
<tr>
<td>12</td>
<td>Internal point situated at the extreme bottom-left corner of the first medial cell</td>
<td>Ⅱ</td>
</tr>
<tr>
<td>13</td>
<td>Point situated at the intersection between the Cu vein and the 1m-cu vein</td>
<td>I</td>
</tr>
<tr>
<td>14</td>
<td>Internal point situated at the extreme bottom-left corner of the second medial cell</td>
<td>Ⅱ</td>
</tr>
<tr>
<td>15</td>
<td>Point situated at the intersection between the Cu vein and the Cu1 vein</td>
<td>I</td>
</tr>
<tr>
<td>16</td>
<td>Internal point situated at the extreme bottom-right corner of the second cubital cell</td>
<td>Ⅱ</td>
</tr>
<tr>
<td>17</td>
<td>Point situated at the intersection between the A vein and the cu-a vein</td>
<td>I</td>
</tr>
<tr>
<td>18</td>
<td>Internal point situated at the extreme top-left corner of the second cubital cell</td>
<td>Ⅱ</td>
</tr>
</tbody>
</table>
Table 2. Floral visitors recorded in the 5 invaded and 5 non-invaded sites in 2017, expressed as proportions. Species in grey were significantly associated with a site-type.

<table>
<thead>
<tr>
<th>Floral visitors</th>
<th>Invaded sites</th>
<th>Non-invaded sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dour 1</td>
<td>Mons 1</td>
</tr>
<tr>
<td><em>Apis mellifera</em></td>
<td>0.12</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Bombus lapidarius</em></td>
<td>0.03</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Bombus pascuorum</em></td>
<td>0.64</td>
<td>0.86</td>
</tr>
<tr>
<td><em>Bombus terrestris</em></td>
<td>0.21</td>
<td>0.14</td>
</tr>
<tr>
<td>Other Hymenoptera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colletes sp.</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Dasypoda hirtipes</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Halictus scabiosae</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Lasioglossum morio</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Lasioglossum sp.1</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Lasioglossum sp.2</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Megachile centuncularis</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Megachile octosignata</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Melitta leporina</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Panurgus calcaratus</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Diptera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erist arbutorum</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Eristalis tenax</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Lepidoptera</td>
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<td>0.00</td>
</tr>
<tr>
<td>Zygaena sp.</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Total number of</td>
<td>33</td>
<td>22</td>
</tr>
</tbody>
</table>

recorded visitors