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Pollen loaf provision in three host plant specialist colletid bees (Hymenoptera, Apoidea, Colletidae)

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Hereby I confirm, that I have prepared the present work independently, exclusively with the use of the here cited literature and utilities. Parts which I copied literally from other publications are clearly indicated as quotations, with declaration of the source.

Mons, 1st March 2013

Contents

1. Abstract

Bees are phytophagous insects. Males and females forage on flowers (nectar and pollen). Female bees provide their offspring with a pollen-nectar mixture. Nectar has high sugar content. Pollen serves as main protein source for bees. Proteins are necessary for larval growth and development and for adult reproduction and longevity. Protein content varies from 12 to 61% in pollen of Angiosperms. However, within plant genera and families the crude protein quantity is highly conserved. How chemical quality of the host plant pollen influence female foraging behavior and offspring size is still under debate. In this study, larval pollen loaves of the sibling species *Colletes halophilus*, *Colletes hederae* and *Colletes succinctus* were analyzed. The three specialist (oligolectic) species differ strongly in their preferred pollen host range. Females of *C. halophilus* forages on Asteraceae, *C. hederae* prefers Araliaceae and *C. succinctus* is specialized on Ericaceae. The influence of pollen quality on female provisioning investment and the influence of pollen loaf quality (i.e. polypeptide quantity) on offspring size were analyzed. Four parameters were examined: (1) size of adult bees, (2) pollen loaf quantity, (3) polypeptide quantity of pollen loaves, and (4) pollen-nectar mixture of pollen loaves. The wing size of the bees was measured in order to estimate the bee size. The fresh and dry weight of adult bees and pollen loaves were measured. Polypeptides of pollen loaf samples were extracted with the aid of phenol and quantified with a BCA Protein Assay Kit. To measure the pollen-nectar mixture the difference between fresh and dry weight of the pollen loaves was considered. The results show that females may invest more foraging time (i.e. larger pollen loaves) in brood cells with female eggs. However they do not vary the polypeptide concentration (i.e. addition of nectar in the pollen-nectar mixture) for male or female larval provision. The female investment is the higher in *C. halophilus* (i.e. the larger pollen loads) and the lesser in *C. succinctus* (i.e. the smaller pollen loaves). Females of *C. hederae* dilute the pollen provision much more with nectar than females of *C. halophilus*. There is no relation between total polypeptide content of pollen and adult bee size.

Key words: Host plant specialization, Colletidae, *Colletes halophilus*, *Colletes hedereae*, *Colletes succinctus*, sexual dimorphism, nesting, brood cells, pollen loaves, oligolecty, polypeptides

2. Introduction

2.1. Bees (Hymenoptera: Apiformes)

2.1.1. Phylogeny

Bees are grouped within the insect order Hymenoptera (*Fig. 1*). They belong to the superfamily Apoidea. Together with the superfamilies Vespoidea (wasps) and Chrysidoidea (ants) they form the section Aculeata. Within the Apoidea there are two groups, the Spheciformes (sphecoid or apoid wasps) and the Apiformes (bees). In contrast to the polyphyletic sphecoid wasps the bees are monophyletic and can be distinguished by several structural characters like the presence of different shaped hairs on the body (e.g. ENGEL 2001, MICHENER 2007). There are 20000 described bee species worldwide (MICHENER 2007).

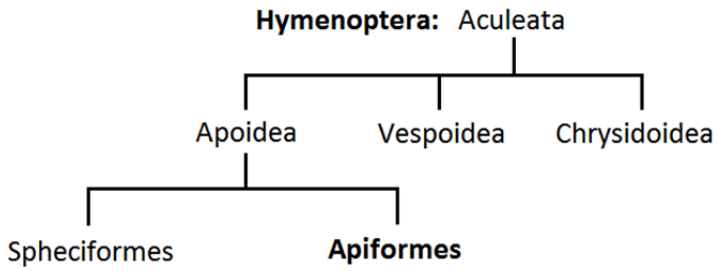

Figure 1. **Classification of bees (Apiformes) within the Hymenoptera.** According to MICHENER 2007, SHARKEY 2007.

The Apiformes are divided into two groups which contain altogether seven bee families (*Fig. 2*). The first group is formed by the long-tongued (LT) bee families Apidae and Megachilidae. The second group is formed by the short-tongued (ST) bee families Melittidae, Andrenidae, Halictidae, Stenotritidae and Colletidae (KIRBY 1802, ENGEL 2001, DANFORTH et al. 2006b). The LT bees are a monophyletic group whereas the ST bees are a paraphyletic group. However the three short-tongued bee families Andrenidae, Colletidae and Halictidae are monophyletic (DANFORTH et al. 2006a, 2013). Within the bees the Mellitidae are the most basal family and the Colletidae are the most derived family (DANFORTH et al. 2006a,b).

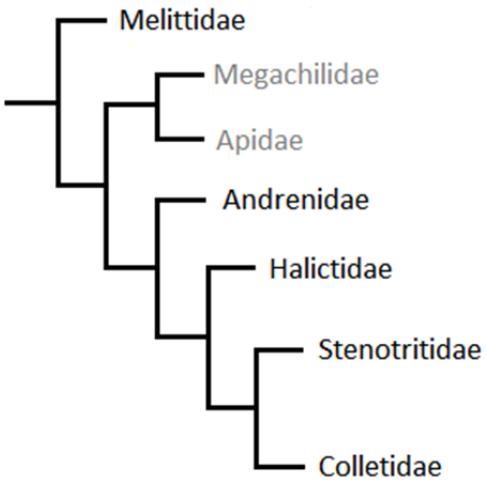

Figure 2. Phylogeny of the families within the Apiformes. LT bees: families written in grey. ST bees: families written in black. According to DANFORTH et al. 2006b, 2013.

2.1.2. Morphology

The morphological characters which distinguish bees from other Hymenoptera concern in general the more efficient powerful flight and adaptions to food collection. That includes the simplification of the wing venation and several adaptions of the thorax, mouthparts and legs (MICHENER 1944).

A body plan according to MICHENER (1944) and STEPHEN et al. (1969) is presented in Figure 3A. Bees are hypognath, the mouthparts are directed ventral-posteriorly. The strong mandibles allow for digging and the maxillae plus labium form a tube which enables the uptake of fluids (i.e. nectar, water). The compound eyes are elongate-oval, convex and located on the lateral parts of the head. The antennal bases are located between the eyes. The antennae of males are longer than the antennae of females; they consist of 11 instead of 10 flagellar segments and the segments are longer than the flagellar segments of females (*Fig. 3B*). The thorax consists of a small prothorax, a large mesothorax and a small metathorax. The first abdominal segment (propodeum) is fused to the metathorax and forms the narrow connection between thorax and the remaining abdomen. The thorax bears the wings (dimorphic fore and hind wings) and the six legs. In non-parasitic female bees the hind legs commonly bear the pollen-collecting apparatus (hairy structures). The apparent abdomen (terminal body region) is called metasoma. It consists of six (females) or seven (males) segments; its first segment is the "true" second abdominal segment (MICHENER 1944, STEPHEN et al. 1969).

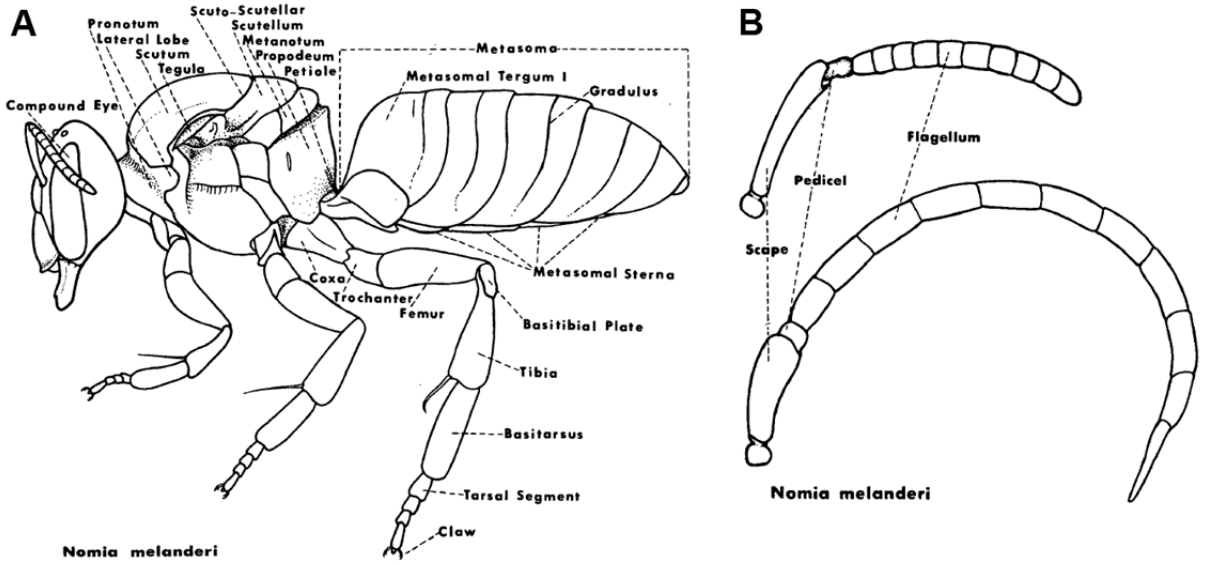

Figure 3. Morphology of a bee. *Nomia melanderi* (Halictidae). From STEPHEN et al. 1969. A: Whole body plan of a bee, wings excluded. B: Antennae of female (above) and male (below) bee.

2.1.3. Life history

The common image of bees is that of colonies of the eusocial honey bee. Within a large nest a single queen lives together with many workers and drones. Only the queen is fertilized and produces offspring which is provisioned and reared by the workers. The division of work contributes to the survival of the collective. However the majority of bees are living solitary. Fertile females construct their own isolated nests, provide nutrition for their offspring and lay eggs. They do not divide work with other bees and do not have contact to their offspring (STEPHEN 1969, MICHENER 2007).

Nearly all nests of solitary bees are composed similarly (*Fig. 4*): one or more brood cells are constructed, provided with pollen and nectar and one or more offspring are reared in every cell (MICHENER 1964, CANE 2008). Most solitary bees nest in the ground and construct their nests following similar steps: excavation of burrows and brood cells, lining of the brood cell walls (in some species), provision with pollen mixtures, oviposition, and closure (MICHENER 1964). Nests can be constructed in the soil, in rotting wood, in pithy stems, in empty snail shells. Some bees use pre-existing holes to construct their brood cells (e.g. Megachilidae). The lining of the brood cells can consist for example of delicate, brown, wax-like (Andrenidae, Halictidae) or cellophane-like, translucent, waterproof material (Colletidae). Some species line their cells with smooth soil before spreading the cell lining. The larval provision can be solid (e.g. Halictidae, Andrenidae) to liquid (Colletidae). The egg is deposited on top of the provision (MICHENER 1964). In some species, females construct brood cells of different size for male and female offspring. It is common that within the Hymenoptera fertilized diploid eggs become female bees and that unfertilized haploid eggs become male bees (e.g. MICHENER 2007). Due to the fact that a female stores the sperm in her spermatheca, she is able to control the sex of each egg. Bees pass through complete

metamorphosis. The adult bee finally emerges and leaves the nest. After mating female bees begin to construct new nests for their own offspring. In temperate regions most species have only one generation per year. Due to this fact the life of the adult bees is very short and the species overwinters as prepupae in their brood cell (STEPHEN et al. 1969, MICHENER 2007).

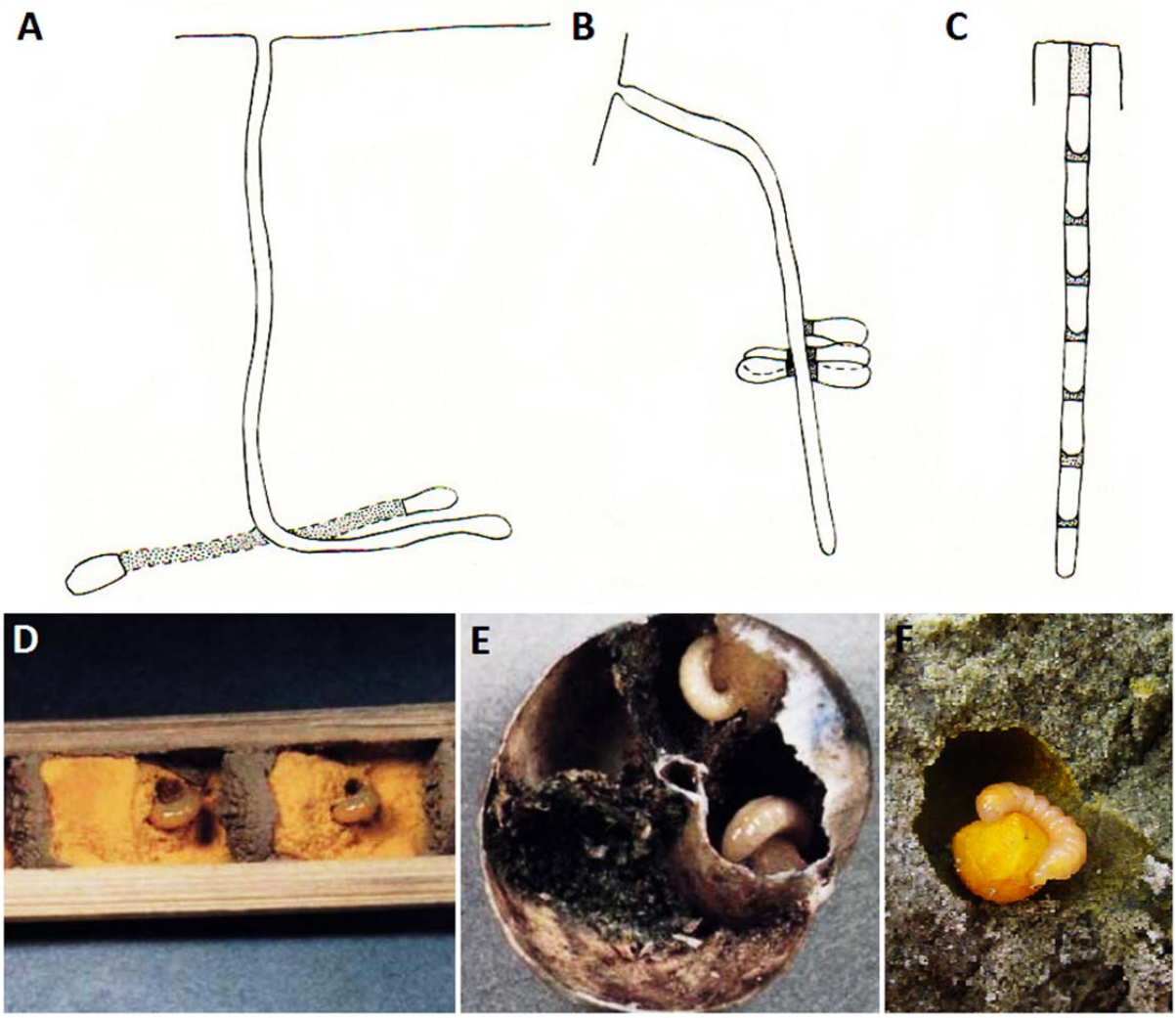

Figure 4. Nest constructions of solitary bees. A: *Andrena erythronii* (Andrenidae) nest in the ground. From MICHENER 1964. B: *Lasioglossum guaruvae* (Halictidae) nest in earth bank. From MICHENER 1964. C: *Hoplitis pilosifrons* (Megachilidae) nest in pithy stem. From MICHENER 1964. D: *Osmia rufa* (Megachilidae) nest in dead bamboo stem. Photo by WESTRICH, from MICHENER 2000. E: *Osmia aurulenta* (Megachilidae) nest in shell of dead snail. Photo by WESTRICH, from MICHENER 2000. F: *Dasypoda hirtipes* (Apidae) nest in the soil. From Laboratoire de Zoologie (UMons).

2.2. Bee-host plant relationships

Bees are one of the most important pollinator group (DANFORTH et al. 2006, MICHENER 2007). While they are foraging on flowers, pollen sticks to them and can fertilize the next flower the bee visits. However, not every bee which forages on a flower is a real pollinator for the plant. Bees also forage on wind-pollinated plant species (ROULSTON et al. 2000). Most of the tree species in tropical rainforests are bee-pollinated; as well as wild flowers, herbaceous plants, several bushes and small trees in the temperate climates. Due to the dramatic decrease in honey bee populations wild bees become more and more important. Their preservation is the requirement for the conservation of many natural vegetative habitats (MICHENER 2007).

Bees are phytophagous. Males and females drink nectar or oil on flowers and eat pollen (MICHENER 2007). To feed their larvae bee females collect pollen and nectar or oil and carry it to the nest. The pollen is transported dry or is mixed with nectar or oils (MICHENER 2007). The common pollen transport devices of bees are the external scopa and the internal crop (*Fig. 5*; e.g. STEPHEN et al. 1969). How many flowers have to be visited to feed one single larva depends on the body size of the bee species and the mass of pollen contained in the flower of the plant (MÜLLER et al. 2006). Müller and colleagues analyzed 41 European bee species and found out that the minimal number of flowers needed to rear one larva varies from less than 10 for the smallest bee species to more than 1000 for very large species.

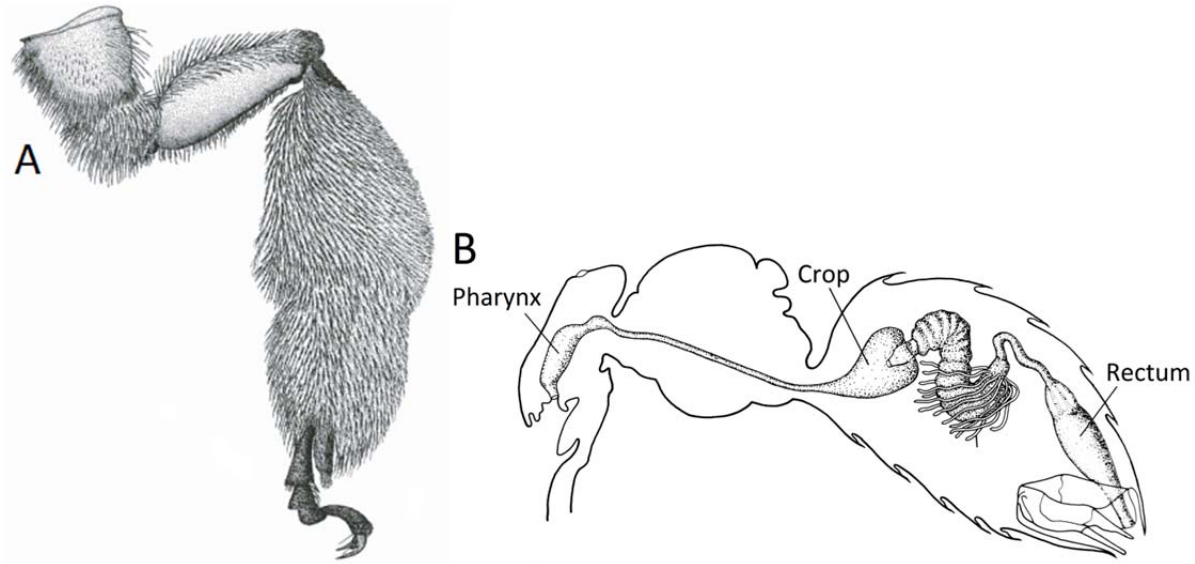

Figure 5. Pollen transport devices. A: Scopa on tibia and basitarsus of hind leg of female *Svastra oblique* (Apidae) (from MICHENER 2000). B: Crop in bee internal anatomy (modified from MEHLHORN 2001).

2.2.1. Host plant specialization

Host plant range of bees is highly variable (MICHENER 2007). Some solitary bees and nearly all eusocial bees are generalists (polylectic) with regard to the flower species they nourish on. Eusocial bees collect nectar and pollen for a long period during the year. For them floral specialization would be unfavorable because most species are in bloom only for a short time.

On the other hand many solitary bees are floral specialists (oligolectic or monolectic). Their periods of foraging activity are short and therefore they can only feed on a few related families or species which are in bloom in the same time (MICHENER 2007). MÜLLER & KUHLMANN (2008) applied the categories and several subcategories concerning flower specialization (*Tab. 1*). In evolutionary point of view oligolecty is considered to be a plesiomorphic state in bees, the most basal clades are narrow specialists (DANFORTH et al. 2006b, MICHEZ et al. 2008).

Table 1. Categories of bee host range. According to MÜLLER & KUHLMANN 2008. s.l. = in the broader sense, s.s. = in the stricter sense.

Category	Subcategories	Definition
Monolecty	-	Pollen collection on only one plant species even in the presence of one or more sympatric species of the same genus. (Pollen collection on only one plant species in the absence of coflowering congenerics is referred to as a special case of narrow oligolecty.)
Oligolecty	Narrow oligolecty	Pollen collection from two to several species belonging to one plant genus.
	Broad oligolecty	Pollen collection from two to several genera belonging to one plant tribe, subfamily or family.
	Eclectic oligolecty	Pollen collection from two to four plant genera belonging to two or three plant families.
Polylecty s.l.	Polylecty with strong preference	Pollen collection from several plant families, but one plant clade (family, subfamily, tribe, genus or species) predominates.
	Mesolecty	Pollen collection from more than four plant genera belonging to two or three plant families.
	Polylecty s.s.	Pollen collection from various genera belonging to at least four plant families.

Bees show several morphological and behavioral specializations which enable nectar and pollen exploitation (MICHENER 2007). Some bees have strong wavy or hooked bristles on their mouth parts (*Fig. 6A*) or on their tarsomera to harvest pollen out of small or very long flower tubes (MÜLLER 1995, 2006). Specialized hairs localized on the face or the underside of the thorax, enable bees to collect pollen from raised anthers (MÜLLER 1996). A stiff pilosity on the hind legs (*Fig. 6B*) is an adaption to remove pollen from inflorescences by pressing together several of the small neighboring flowers and pulling up the pollen with the hind legs (MÜLLER & BANSAC 2004). With specialized hairs on the ventral abdomen bees detach pollen from flower heads by rapidly moving the abdomen (MÜLLER 2008). Another behavioral adaption to harvest pollen is the "buzzing" of anthers with vibration movements of the flight muscles (e.g. CANE 2008). In general bees that are polylectic show a higher flexibility in using different organs for the pollen uptake from flowers of different shape (MÜLLER 1996).

But specialists are able to harvest pollen faster and more efficient because they are better adapted to the morphology of their host plant (WESTERKAMP & CLAßEN-BOCKHOFF 2007). Due to their morphological specialization bees are able to switch to new host-plants which show similar floral morphology, although they are not related to the typically visited plant family (e.g. MICHEZ et al. 2008).

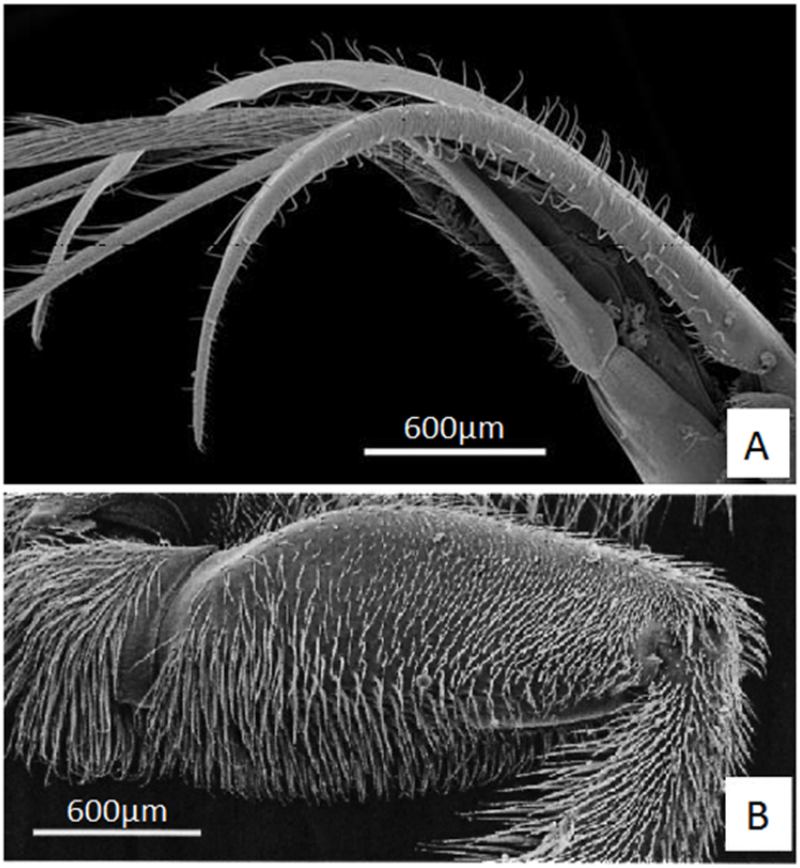

Figure 6. Pollen harvesting devices. A: Mouthparts of *Hoplitis pici* (Megachilidae) with pollen harvesting bristles (from MÜLLER 2006). B: Hind leg brush on ventral side of trochanter and femur of *Megachile apicalis* (Megachilidae) as seen from behind (from MÜLLER 2004).

To ensure pollination by bees, flowers have to be highly recognizable. Besides the shape and color of the flower, floral scent (TOLLSTEN & KNUDSEN 1992) is useful for the attraction over long distances (DÖTTERL & VEREECKEN 2010). The floral compounds can be perceived by the antennae of bees and therefore play a key role in the attraction of these pollinators (DÖTTERL et al. 2005). Several floral scent compounds and attracted bees were listed by DÖTTERL & VEREECKEN (2010). However for the plant it is important to find a balance between the attraction of bees (i.e. pollen exploitation) and the conservation of the pollen for reproduction (MÜLLER & KUHLMANN 2008, SEDIVY et al. 2011). Pollen grains which are collected by female bees can not be used for plant reproduction (WESTERKAMP & CLAßEN-BOCKHOFF 2007). MÜLLER et al. (2006) claimed that one female bee can reap approximately 40% pollen of one flower during one visit. Due to the fact that a flower is visited by not only one bee, the loss of pollen can be 70% up to 95% (THOMSON 2003, SCHLINDWEIN ET AL. 2005).

There are morphological adaptions concerning the shape of the flowers or anthers which allow pollen collection only for a small number of specialized bee species. The gradually release of pollen by the anthers (CASTELLANOS et al. 2006) ensures that bees can not harvest all the pollen. Furthermore pollen can be protected chemically by compounds which are toxic for female bees and their larvae (RHARRABE et al. 2010, SEDIVY et al. 2011). For example toxins or pigments in the oily pollenkit that sticks together the pollen; alkaloids or phytoecdysteroids which are homologous to the ecdysteroids in insects. Unfavorable or protective properties which require physiological adaptions of bees are present in pollen of plants from the families Asteraceae, Ranunculaceae, Brassicaceae, and Boraginaceae (DINAN 2001, MÜLLER & KUHLMANN 2008, PRAZ et al. 2008, SEDIVY et al. 2011).

2.2.2. Pollen content

Plants provide adult bees and larvae with all necessary nutritive substances (i.e. sugar, protein, amino acids, sterols and minerals). Oil is a useful source for calories (WCISLO & CANE 1996). Nectar mainly contains sugars. The mean concentration is approximately 30%, but ranges from 10% to 70% (RASMONT et al. 2005). However nectar contains a low mineral matter and only traces of amino acids and sterols (RASMONT et al. 2005). Pollen is the main source of proteins and lipids. Pollen contains 2.5 to 61% crude protein (of dry mass) (BUCHMANN 1986). In general all common amino acids are present in pollen (ROULSTON & CANE 2000). The starch content of pollen ranges from 0 to 22% (ROULSTON & CANE 2000). Fat soluble compounds like fats, fatty acids, some vitamins, pigments, higher alcohols, waxes, sterols and saturated hydrocarbons represent 1 to 20% of pollen weight (ROULSTON & CANE 2000). Δ5-avenasterol and β-sitosterol are the most frequent sterols in pollen whereas the amount of cholesterol is very small (RASMONT et al. 2005). Pollen further contains approximately 3.5% minerals, especially Ca, K, and Mg. Also present are Na, Fe, Zn, Mn, and least Cu (RASMONT et al. 2005).

2.2.3. Proteins

Proteins are polypeptides which consist of chains of hundreds of amino acids which are connected by peptide bonds (*Fig. 7A*, CAMPBELL & REECE 2004). Due to the sequence of the amino acids and their interactions the steric conformation of the protein is defined (*Fig. 7B*, BERG et al. 2002). Proteins have several functions and are essential for all biological processes. Proteins can be composed of 20 different amino acids (BERG et al. 2002). Essential amino acids can not be synthesized by an organism but have to carry by nutrition (BERG et al. 2002, CAMPBELL & REECE 2004).

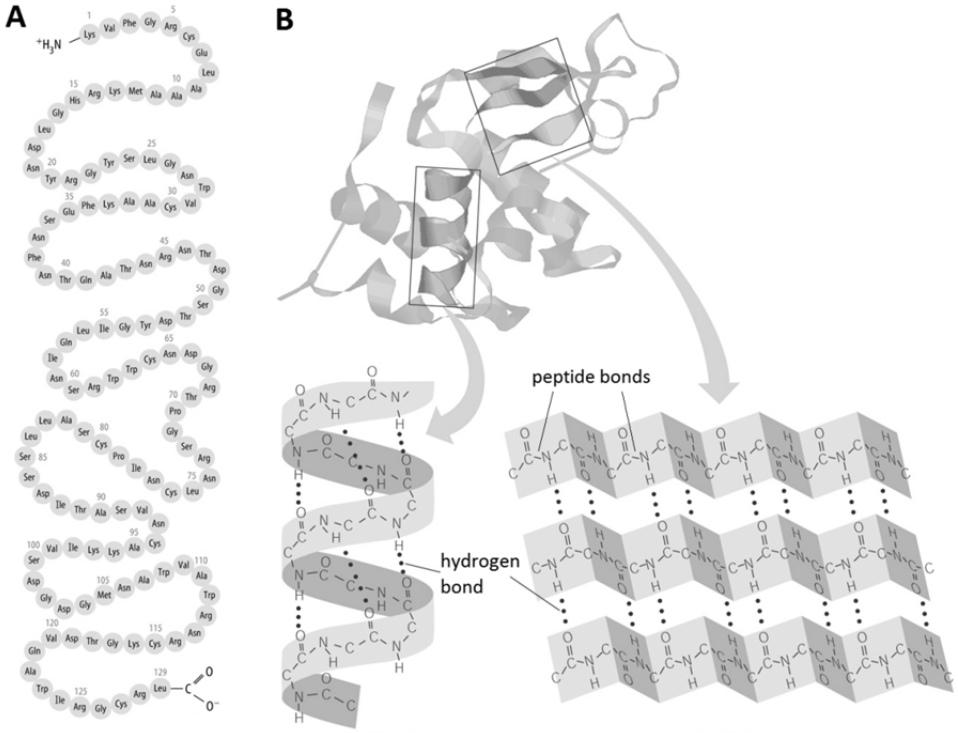

Figure 7. Structure of a protein. A: chain of amino acids connected by peptide bonds (lysozyme enzyme). B: Steric conformation in consequence of hydrogen bonds. Modified from CAMPBELL & REECE (2004).

NATION (2002) summarized the utilization of proteins by insects. Most insect species need particular dietary protein contents for optimal growth and development. For female adult insects proteins are especially required for the synthesis of the juvenile hormone and the development of ovaries and eggs. The male sperm development does not require proteins. The nutritional protein is the source of amino acids. Essential amino acids which can not be synthesized by insects are: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine. Optimal growth and development however requires a mixture of essential and non-essential amino acids, due to the fact that the synthesis of amino acids requires vast amounts of energy (NATION 2002).

As for other insects, proteins are necessary for the development and reproduction of bees. Further longevity of adult bees can increase when they forage on protein-rich pollen (ROULSTON & CANE 2002). There is no evidence that bees prefer to collect protein-rich pollen (ROULSTON et al. 2000). However the pollen protein concentration may influence the number of foraging trips that female bees make in order to provide their offspring with enough protein. The protein concentration of the pollen diet and the amount of pollen which is consumed determine the total protein which can be consumed (ROULSTON & CANE 2000). Thus the offspring size of bees may correspond to provision size and nutrient content of the provision. For the bumblebee *Bumbus terrestris* it was found that a pollen diet rich in polypeptides leads to the production of more or larger larvae (VANDERPLANCK et al., in press). The authors suggest that *B. terrestris* balance low nutrition (i.e. polypeptide) contents with larger syrup consumption. For *Lasioglossum zephyrum* it was also reported that the offspring body size is related to total protein consume. Protein-poor pollen leads to smaller offspring

(ROULSTON & CANE 2002). Furthermore the authors found that the provision size for *L. zephyrum* offspring was not adjusted to the pollen protein content: the protein amount in the provision increased with increasing protein concentration in the host pollen (ROULSTON & CANE 2002).

In general animal-pollinated plant species do not have pollen with higher protein amounts than wind-pollinated species (ROULSTON et al. 2000). All common amino acids are present in pollen (ROULSTON & CANE 2000). Most pollen contain all essential amino acids and pollen of related plant species have very similar proportions of amino acids (WEINER et al. 2010). However, the essential amino acids phenylalanine and tryptophane are sometimes only present in traces or even lacking (ROULSTON & CANE 2000, RASMONT et al. 2005, WEINER et al. 2010). Pollen of plant species visited by oligolectic bees show a significantly lower pollen quality (i.e. lower concentration of total amino acids and total essential amino acids) (WEINER et al. 2010). The crude protein concentration of angiosperm pollen ranges from 12 to 61% but is highly conserved within plant genera and families (ROULSTON et al. 2000).

2.3. Objectives

The main objective of this study is to examine particular behavior of the females of three sister species of solitary bees. Do they adapt pollen provision for the offspring (a) to their own size, (b) to the sex of the deposited egg, and (c) to the pollen quality?

The following questions will be considered:

(1) Are there intraspecific or interspecific differences in the size, weight and ratio of size and weight between the adult bees?

(2) Are there intraspecific or interspecific differences in the female investment for the pollen provision of their offspring (quantity of pollen loaf, protein concentration, protein quantity, pollen quantity)?

(3) Are there intraspecific or interspecific differences in pollen loaf quality and size related to emerging adults?

2.4. Biological model - Colletidae

2.4.1. Phylogeny and characteristics

The Colletidae are a monophyletic family of the Apiformes (BRADY & DANFORTH 2004, DANFORTH et al. 2006a,b, ALMEIDA & DANFORTH 2009). For a long time they were supposed to be the most ancient family within the Apiformes, due to a high similarity of the colletid mouthparts with those of apoid (spheciform) wasps, the sister group to bees (e.g. Michener 1964, words ENGEL 2001 p.23: "[The glossa is] broadened or usually bilobed at its apex as in the spheciform wasps"). A comparison of several molecular and morphological data proved that Colletidae are not the most basal family of the bees but a highly derived bee family (*Fig. 8*; DANFORTH et al. 2006a+b, 2013). The Colletidae comprise approximately 2500 species (ALMEIDA & DANFORTH 2009, ALMEIDA et al. 2012)

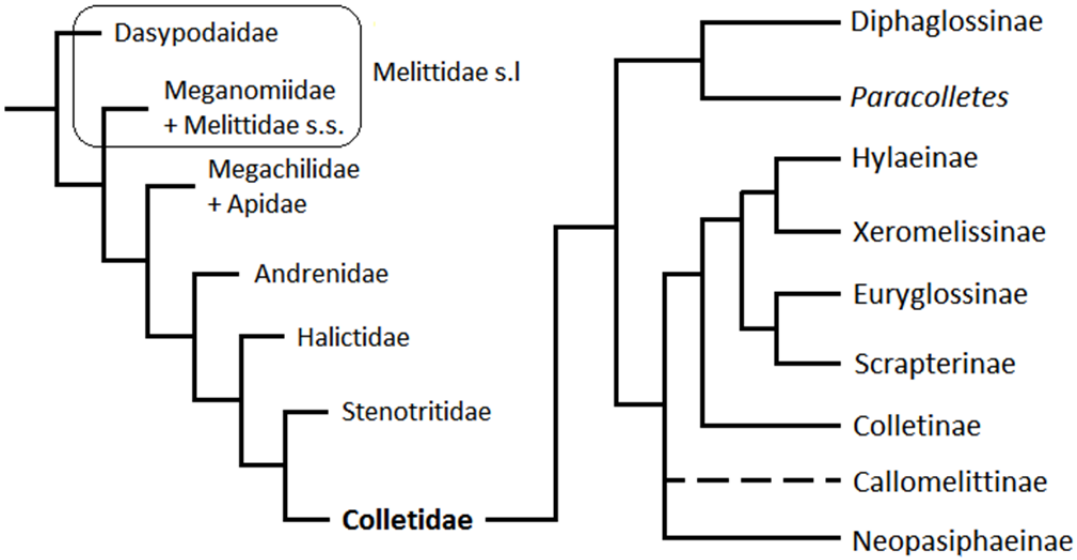

Figure 8. Phylogeny of Colletidae. Left: Position of the Colletidae within the Apiformes. According to DANFORTH et al. 2006b, DANFORTH et al. 2013. Right: Phylogeny of the families within the Colletidae. According to ALMEIDA et al. 2012, DANFORTH et al. 2013.

The monophyly of the Colletidae is supported by several parameters: (1) by molecular data (DANFORTH et al. 2006a+b, ALMEIDA & DANFORTH 2009), (2) by the typical shape of their mouthparts (short, bilobed brush-like glossa), what distinguishes them from all other bees (MICHENER 2007, ALMEIDA 2008), and (3) by the unique cell lining of their nests (e.g. MICHENER 2007; ALMEIDA 2008).

The majority of colletid species are living solitary (MICHENER 2007, ALMEIDA 2008). Most characteristic for Colletidae are the truncate bilobed or bifid glossa (*Fig. 9*), and the strongly arcuate second recurrent vein on the forewings (*Fig. 10*) (MICHENER 1944, STEPHEN et al. 1969, MICHENER 2000). To collect pollen most colletid females have large scopae which range from the coxa to the basitarsus of their hind legs (MICHENER 2007). Only females of Euryglossinae and Hylaeinae transport pollen internally in the crop (e.g. MICHENER 1964, ALMEIDA 2008).

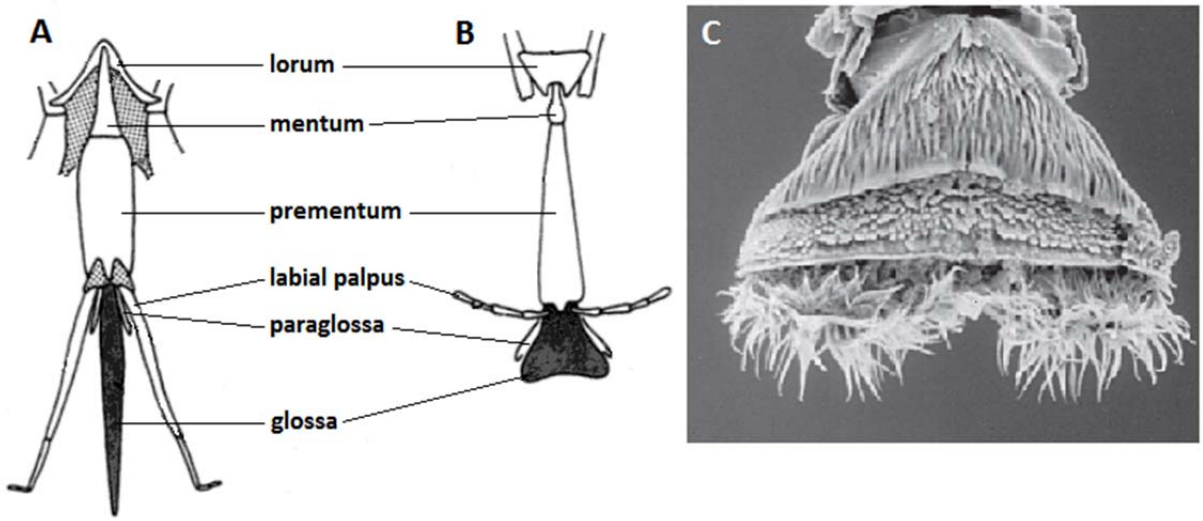

Figure 9. Glossa within the mouthparts. A: Long tongued bee (modified from MICHENER 2000). B: Short tongued (colletid) bee (modified from MICHENER 2000). C: SEM photo of the bilobed glossa of the colletid bee *Hylaeus basalis* (from ALMEIDA 2008).

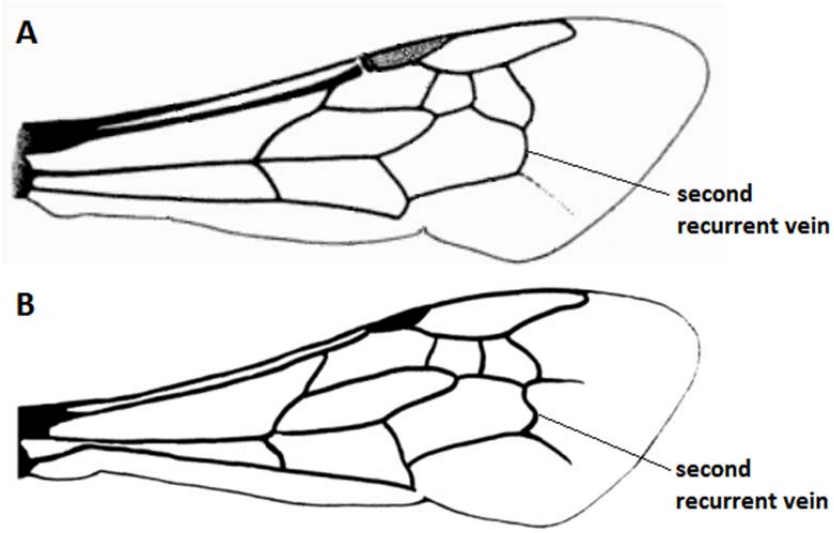

Figure 10. Right anterior wing. A: General structure. Second recurrent vein lightly curved (modified from Michener 2000). B: Structure of colletid bee wing. Second recurrent vein strongly arcuate (drawn according to photograph of male *C. succinctus* wing, 2012).

Most colletid species are distributed to the southern hemisphere, especially Australia, southern Africa and South America (*Fig. 11*). Only the two genera *Colletes* and *Hylaeus* can be found in the Holarctic region (MICHENER 2007, ALMEIDA & DANFORTH 2009, ALMEIDA et al. 2012). An explicit overview of the biogeography and diversification of the Colletidae was given by ALMEIDA et al. (2012). The origin of this family is in the southern hemisphere on the former supercontinent Gondwana. Distribution and diversification of the colletid bees initiated only after the African and the South American continents were separated, but while Australia and South America still were connected with each other by Antarctica. Following diversification and differentiation between Australian and South American lineages could take place due to the detachment of Antarctica.

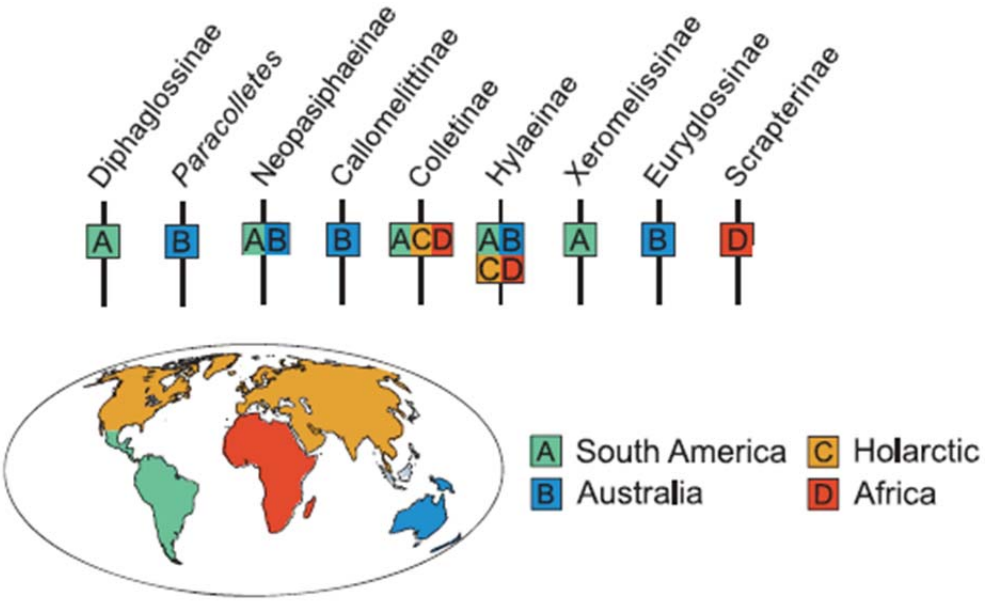

Figure 11. Distribution of Colletidae. Summary modified from ALMEIDA et al. 2012.

The nesting behavior of Colletidae was reviewed by ALMEIDA (2008). Colletidae nest in stems, dilapidated wood, volcanic rock, brittle walls or in the soil (e.g. most Colletinae). Aggregations of several nests are characteristic for Colletidae. Female bees of ground-nesting species excavate a main burrow and lateral burrows. The lateral burrows either end up in a single brood cell or in several consecutive brood cells (in some species of the genus *Colletes*). To protect their offspring against fungi, bacteria, inundation or desiccation colletid bees line their brood cells with a multilayered cellophane-like polyester material (see also MICHENER 1964, HEFETZ et al. 1979, ALMEIDA & DANFORTH 2009). This material is translucent to transparent, waterproof and can contain silk-like filaments (MICHENER 1964). The cell lining material contains secretions of the Dufour's gland (see *Fig. 12*; ALBANS et al. 1980, HEFETZ 1987, MICHENER 2007). It is spread to the brood cell wall by the mouthparts and then polymerizes (see also MICHENER 2007). Most female Colletidae provide their offspring with a liquid to semi-liquid pollen nectar-mixture. One egg is deposited on top of the provision (*Fig. 13A*) or fixed to the cell lining above the provision (*Fig. 13B*; e.g. *Colletes*). The brood cell is closed by layers of the polyester material and a substrate plug (MICHENER 1964). From each brood cell one mature bee emerges (*Fig. 14*).

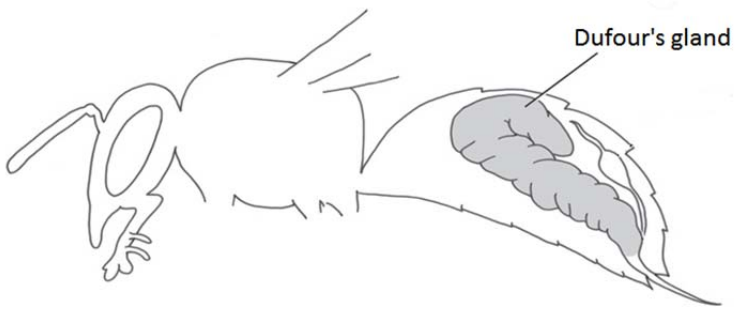

Figure 12. Position of the Dufour's gland in a female *Colletes.* From: ALMEIDA 2008.

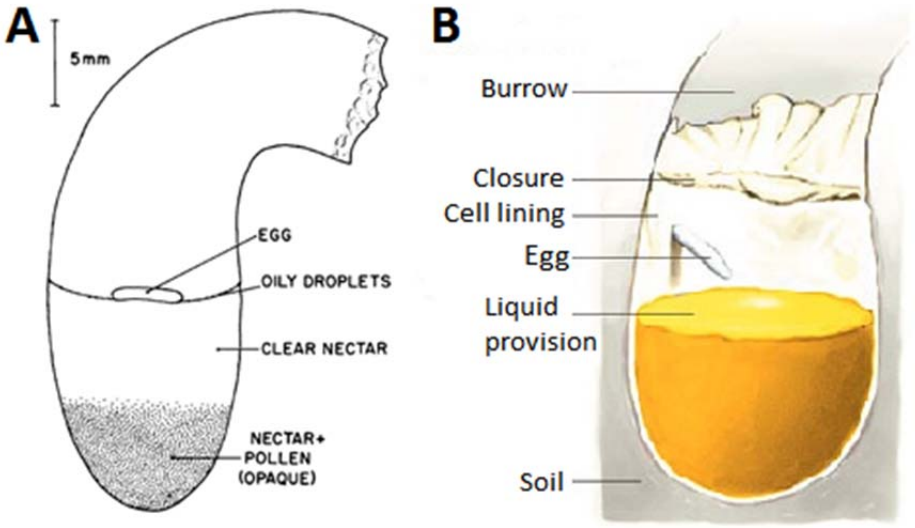

Figure 13. Different deposition of colletid egg. A: Brood cell of *Ptiloglossa arizonensis* (Diphaglossinae). The egg is positioned on top of the provision. From ROZEN 1984. B: Brood cell of *Colletes inaequalis*. The egg is detached to the wall of the brood cell above the pollen provision. Modified from drawing of P. CLARK (2011, The Washington Post).

Figure 14. Exhausted brood cell with freshly emerging bee. *Colletes halophilus*. Scale bar 10mm. Photo: Karolin Schneider, Netherlands (Saefthinghe), 2012.

TORCHIO et al. (1988) observed females of colletid bees (*Colletes kincaidii*) constructing several nests in provided horizontal glass tubes in their laboratory (*Fig. 15*). The females lined the cell (i.e. glass) wall by repeatedly brushing salivary and Dufour's gland material on the whole surface (*Fig. 15A*). The layers for the cell closure were prepared before providing the cell with pollen and nectar (*Fig. 15B*). TORCHIO could observe that the female first brings pollen into the cell and moistens it later with nectar (*Fig. 15C,D*). After 10 provisioning trips the female deposited the egg to the upper cell wall (*Fig. 15E*) and closed the brood cell (*Fig. 15F*).

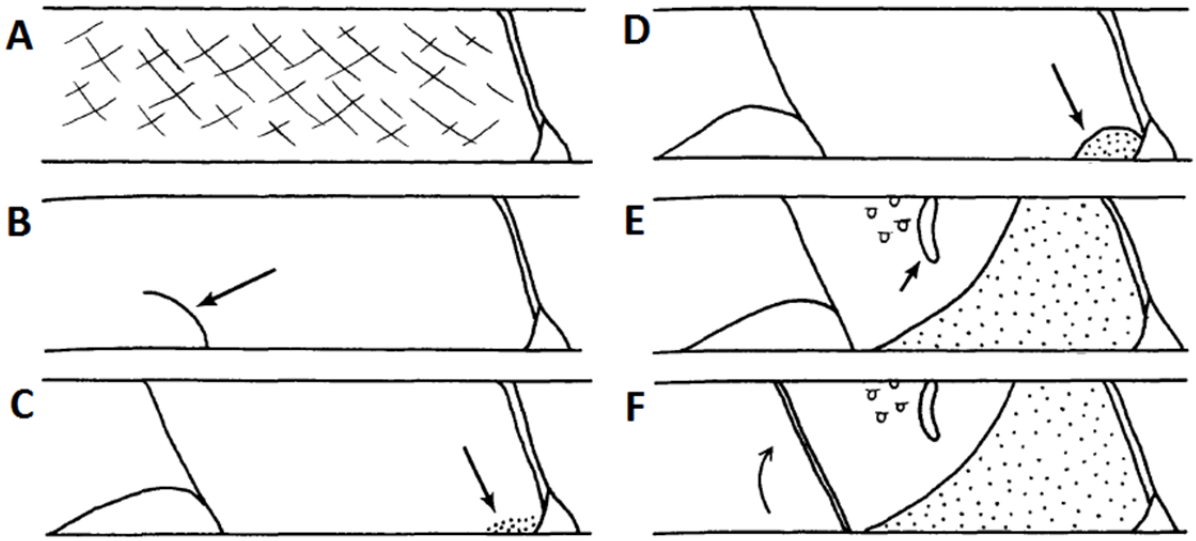

Figure 15. Schematic diagrams showing sequence of cell provisioning, egg deposition and cell closure by *Colletes kincaidii*. Modified from TORCHIO et al. 1988. A: Cell cap for the new cell is constructed and all cell walls are lined. B: The layer for the cell closure is prepared. C: Dry pollen is filled into the cell. D: Pollen is moistened with nectar. E: After 10 provisioning trips the egg is deposited and detached to the upper cell wall. F: Closure of the cell.

2.4.2. Genus *Colletes* Latreille, 1802

The genus *Colletes* was first described by LATREILLE in 1802. Together with the genera *Hemicolletes*, *Rhynchocolletes*, and *Xanthocolletes* it forms the subfamily Colletinae (DANFORTH et al. 2013).

Most species of *Colletes* have pale bands of hair on the posterior margin of the tergites of the otherwise black abdomen. Furthermore they can be distinguished by the cubital cells of the wings. The first submarginal cell is approximately as long as the second and the third submarginal cells together (*Fig. 16*; MICHENER 1944).

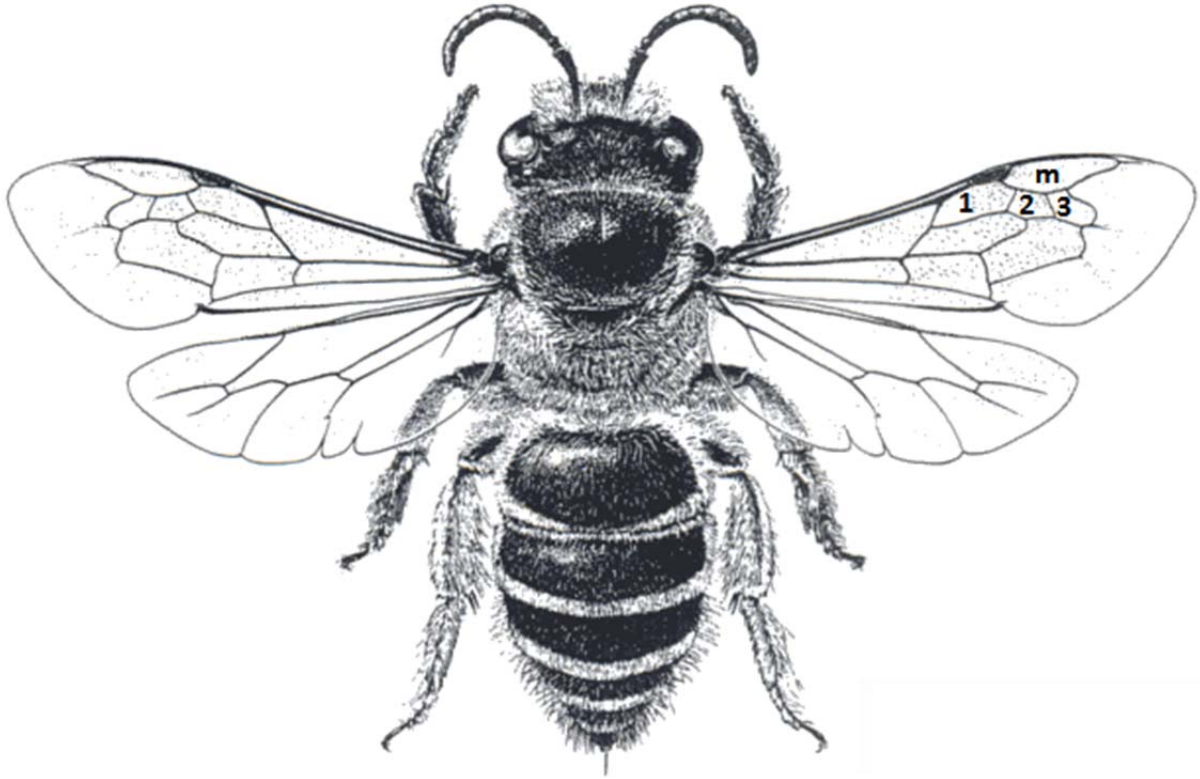

Figure 16. Drawing of *Colletes* dorsal morphology. Female *Colletes cercidii*. The typical pale bands on the abdomen are clearly visible. The first submarginal cell (1) is as long as the second (2) and the third (3) submarginal cells together. m=marginal cell. Drawing by E.R.S. HODGES; from MICHENER 2000).

The genus *Colletes* contains 469 described species and is distributed to the Palearctic and Oriental (205 species), Afrotropical (62), Nearctic (107) and Neotropical (95) region (KUHLMANN 2011 Checklist *Colletes*). It is absent in Australia, Antarctica, Madagascar, tropical Southeast Asia and some oceanic islands. The genus originates from South America and spread to North America and the Eastern Hemisphere. At last the African parts south of the Sahara were colonized (KUHLMANN et al. 2009).

The Palearctic and Oriental species of the genus *Colletes* are divided into 30 taxonomic groups. One of these groups is the *Colletes succinctus*-group. This group exists of 14 described species (KUHLMANN 2011 Checklist Pal+Ori). Species of this group belong to the subgenus *Colletes* in the narrow sense (s. s.) (see *Fig. 17*; KUHLMANN et al. 2009).

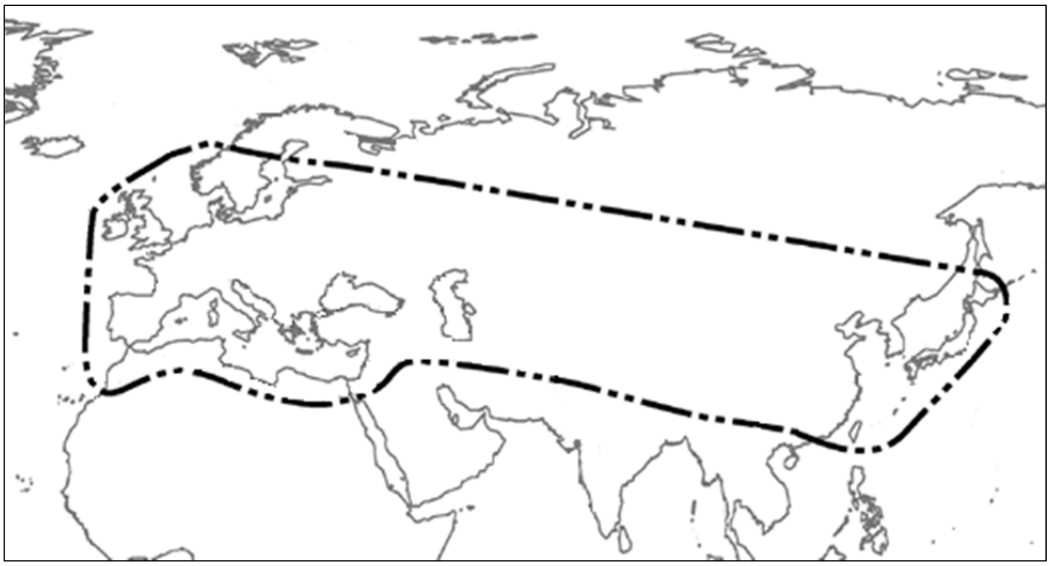

Figure 17. Distribution of the subgenus *Colletes* s.s. Modified from KUHLMANN et al. 2009.

The morphological differentiation of species included in the *succinctus*-group is very difficult. Females are distinguishable by subtile differences in the punctation of tergites, the shape of clypeus and galea and the hairiness. Males show little but characteristically differences in the shape of sternite 7 (KUHLMANN 2003, MICHENER 2007). The three herein studied species *Colletes halophilus* Verhoeff 1944, *Colletes hederae* Schmidt & Westrich 1993, and *Colletes succinctus* (Linnaeus 1758) belong to the *Colletes succinctus*-group. They are closely related, morphologically extremely similar and can only be distinguished by considering molecular data, biogeography, phenology (flight activity, see *Fig. 18*) and flower specialization (KUHLMANN et al. 2007). According to KUHLMANN et al. (2007) it is supposed that *C. halophilus* and *C. hederae* have evolved from *C. succinctus*.

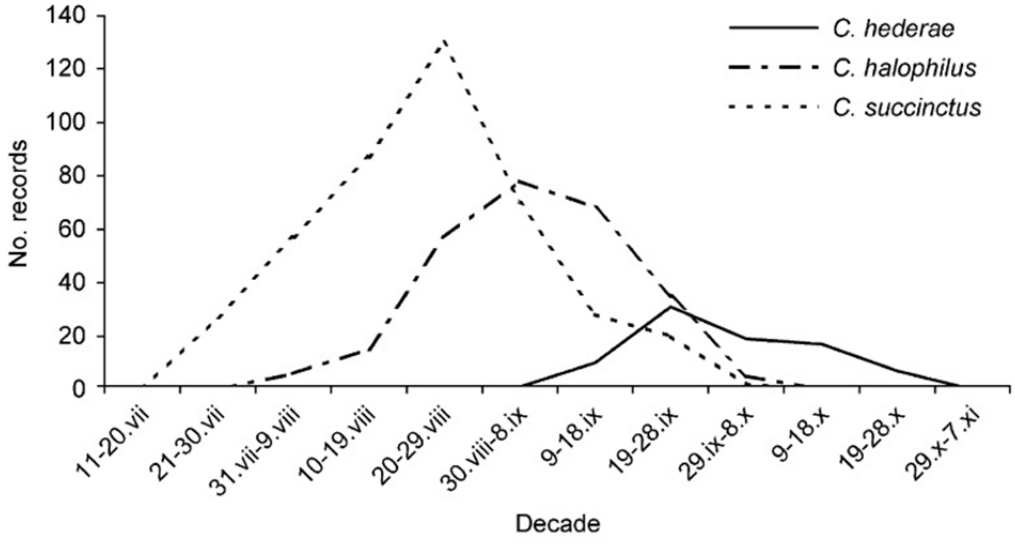

Figure 18. Phenology of *Colletes halophilus*, *Colletes hederae* and *Colletes succinctus*. Based on records from the Netherlands and Britain (KUHLMANN et al. 2007).

Colletes halophilus Verhoeff 1943

Originally *C. halophilus* was considered to be *C. succinctus*. In 1943 it was firstly described as distinct taxon, but as sub-species of *C. succinctus* (VERHOEFF). One character to distinguish the two very similar species is the galea which is mat and structured in *C. halophilus* but shiny in *C. succinctus* (SCHMIDT & WESTRICH 1993). Females of *C. halophilus* are 12-14mm long and males are 11-12mm long (VERHOEFF 1943).

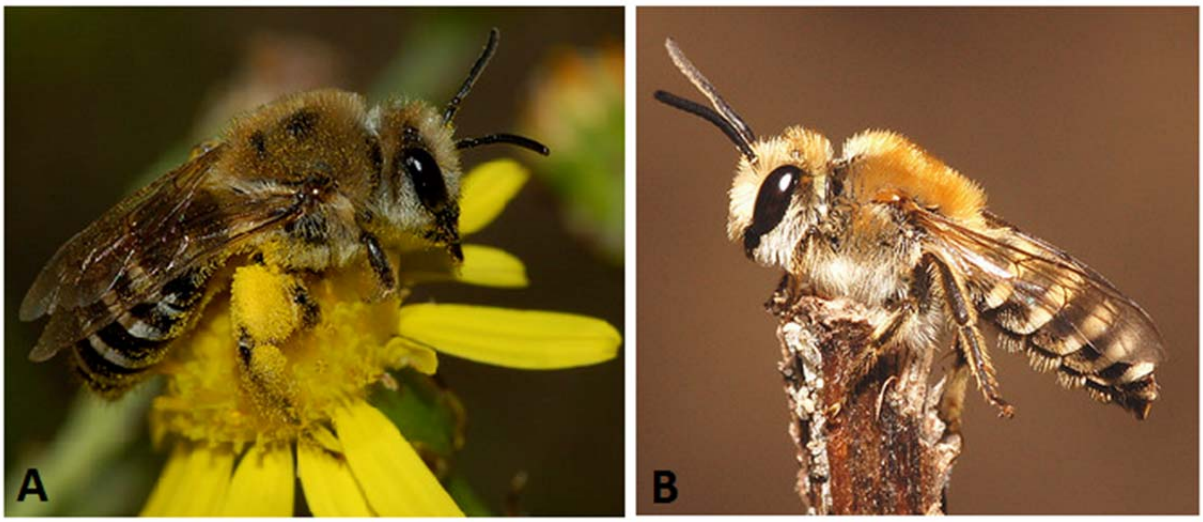

Figure 19. *Colletes halophilus*. A: Female foraging on *Senecio inaequidens* (Asteraceae). B: Male. Photos: Nicolas VEREECKEN.

C. halophilus is an oligolectic species on Asteraceae. Its main pollen source is *Aster tripolium* (*Fig. 20*), but it collects also minor quantity of pollen on other Asteroideae, Brassicaceae and Fabaceae (e.g. WESTRICH 1989, Müller & Kuhlmann 2008, SOMMEIJER et al. 2009).

Figure 20. *Aster tripolium*. From Wikipedia.org.

The distribution of *C. halophilus* is restricted to the coasts of the southern North Sea and the English Channel (*Fig. 21*). It can be found in the south-east of England from the north-west Germany to north-west France (KUHLMANN et al. 2007).

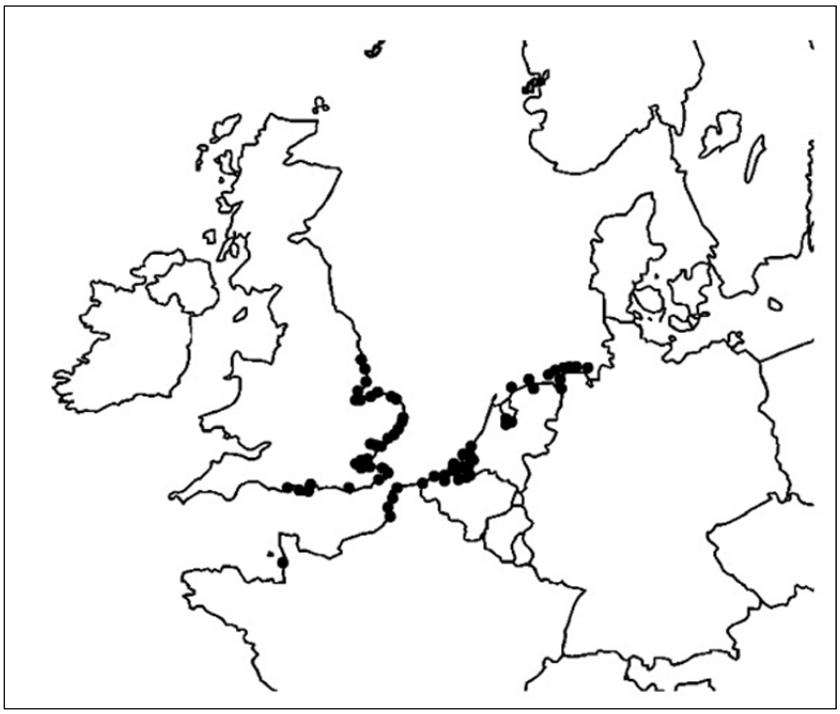

Figure 21. Distribution of *Colletes halophilus*. Black dots indicate the locations of *C. halophilus*. From KUHLMANN et al. 2007.

C. halophilus can be seen from mid-August until early November (see *Fig. 18*; KUHLMANN et al. 2007). *C. halophilus* nests in very different habitat types in coastal areas or saltmarshes (e.g. KNOWLES 2011). Nests were found in horizontal ground, moderate slopes and vertical slopes. The nesting substrates range from sand and gravel, to loam or marine alluvium (KNOWLES et al. 2011). *C. halophilus* nests in large aggregations. One nest consists of several brood cells (*Fig. 22*). The cell lining of *C. halophilus* brood cells has a thickness of approximately 30µm (BELISLE et al. 2011). It consists of two components: a protein-less matrix and proteinaceous silk fibers (as observed by MICHENER 1964) on the outer surface. The silk fibers may play a role as scaffold and may lead to the polymerization of the matrix. Furthermore BELISLE et al. (2011) suppose a salivary gland origin of the silk fibers (see also ALBANS et al. 1980).

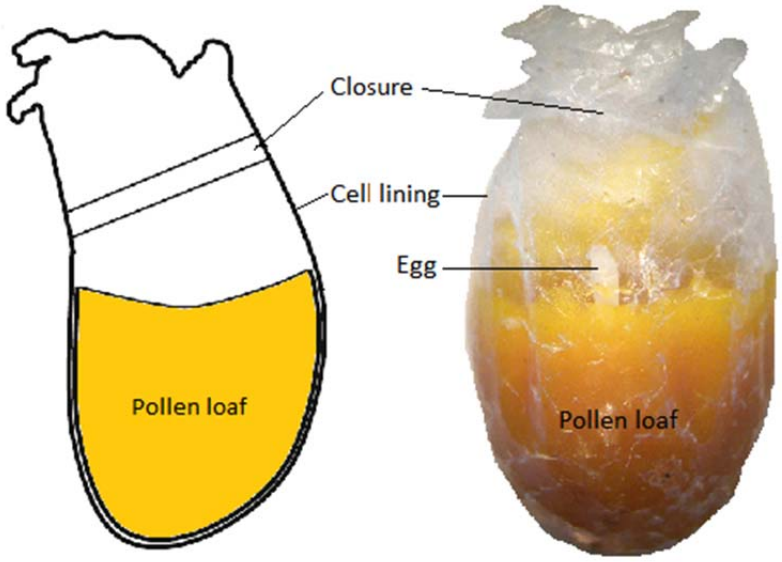

Figure 22. Brood cell of *Colletes halophilus*. Diagramed brood cell (modified from ROOIJAKKERS & SOMMEIJER 2009) and natural brood cell with an egg inside (Photo: Karolin Schneider, collected in the Netherlands, Saefthinghe, 2012).

The brood cells of *C. halophilus* vary in their size depending on the sex of the egg. According to ROOIJAKKERS & SOMMEIJER (2009) cells which contain female eggs, on average have a height of 13.5mm, a diameter of 7.3mm and a weight of 266.8mg. Cells with male eggs are on average 12mm high, 6.7mm wide and have a weight of 185.5mg. That indicates that females of *C. halophilus* are aware of the sex of their offspring while digging the nest and constructing the brood cells. However the variability of the size of cells with male eggs is much higher than in cells with females. ROOIJAKKERS & SOMMEIJER (2009) concluded that the female bee is able to change her decision and put a male egg into a "female" brood cell, when the conditions are not appropriate for a female egg. SOMMEIJER et al. (2009) analyzed pollen loaves of *C. halophilus*. The pollen loaves contain a minimum of 92% of *Aster tripolium* pollen and a small amount of Asteracae and Brassicacae pollen in the solid component (i.e. pollen). The liquid component (i.e. nectar) contains a sugar concentration of approximately 46.5% (mainly glucose and fructose). Furthermore the pollen provision showed hydrogen peroxide production which may originate from glucose oxidase which is provided by the female *C. halophilus*.

Colletes hederae Schmidt & Westrich 1993

In the past individuals of *C. hederae* were wrongly considered to be either an individual of the species *C. halophilus* or *C. succinctus*, due to the high similarity to both species. In 1993 SCHMIDT & WESTRICH firstly described *C. hederae* as discrete species. The females of *C. hederae* are 11.5 to 14.5mm long and therefore much larger than *C. succinctus*. Males are 8.5 to 12.7mm long. Both sexes can be distinguished from *C. halophilus* for example due to the dotting of the tergites or the hairy bands of the tergites, which are brownish in freshly emerged individuals of *C. hederae*, but white in *C. halophilus*.

Figure 23. Female of *Colletes hederae*. Foraging on *Hedera helix*, with pollen loads on the hind legs. (Photo Nicolas Vereecken: VEREECKEN et al. 2009)

Figure 24. Male of *Colletes hederae*. Foraging on *Hedera helix*. (Photo Nicolas Vereecken: VEREECKEN et al. 2006)

C. hederae is polylectic, but shows a strong preference for the Araliaceae *Hedera helix* (see *Fig. 25*, MÜLLER & KUHLMANN 2008). In the past the species was supposed to be oligolectic on *Hedera helix* (SCHMIDT & WESTRICH 1993, BISCHOFF et al. 2005). However the analysis of *C. hederae* pollen loads showed that females collect also pollen on other plants like Asteraceae (Cichorioideae, Asteroideae), Ericaceae (*Calluna*), Orobanchaceae (*Odontites*), Fabaceae and Colchicaceae (*Colchium autumnale*) (WESTRICH 2008, MÜLLER & KUHLMANN 2008). It was shown that especially at the beginning of their flight period, when *Hederae helix* is not completely in flower, pollen loads of the females consisted of 87% Asteraceae, Fabaceae and Colchicaceae (*Colchium autumnale*) (WESTRICH 2008).

Figure 25. Flowers of *Hedera helix* (Araliaceae). Photo: Karolin Schneider, October 2012, Belgium, Brussels (Koekelberg).

C. hederae is widely distributed in the south west Europe (*Fig. 26*). Its distribution ranges from the coast of the Thames, the English Channel and the coast of Belgium and small coastal parts of the Netherlands in the north, to southern Spain. In the west its distribution ranges to the Atlantic Ocean. The eastern border of its distribution is south-west Germany, the coasts of Slovenia and Croatia and the eastern coast of Greece (VEREECKEN et al. 2009). *C. hederae* is distributed where its preferred host plant *Hedera helix* is available. However *Hedera helix* has a much further distribution to east and north Europe (see *Fig. 26*).

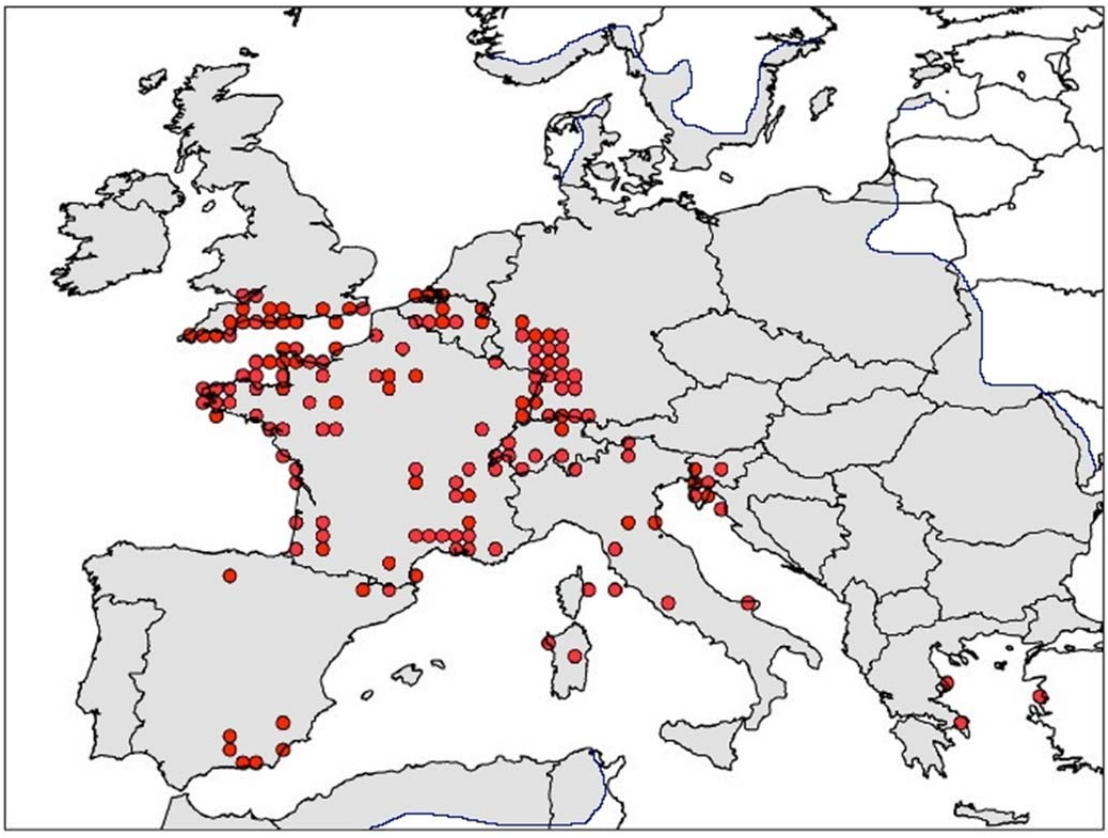

Figure 26. Distribution of *Colletes hederae*. Red dots indicate locations of *C. hederae* (VEREECKEN et al. 2009). The grey shaded area indicates the distribution of *Hedera helix* (according to KUHLMANN et al. 2007).

It is supposed that *C. hederae* has a relatively recent origin because: (1) its distribution seems to be a result of the postglacial recolonization of Europe, (2) its favored host plant *Hedera helix* is distributed much further, and (3) because of its high morphological similarity to its sibling species *C. halophilus* and *C. succinctus* (BISCHOFF et al. 2005).

Simultaneously to the blooming period of *Hedera helix C. hederae* appears in the autumn and can be active until November (see *Fig. 18*; SCHMIDT & WESTRICH 1993, KUHLMANN et al. 2007). A detailed study of flight activity, nest architecture and diurnal foraging rhythm was carried out by BISCHOFF et al. (2005). Males appear some days before the females. After mating, the females start to dig their nests; preferential in sunny inclined slopes with small vegetation. Thereby large nest aggregations are established. Females dig a main burrow and construct several brood cells at the end of this burrow (*Fig. 27A*). The brood cells are approximately 1 to 1.5cm long and have a diameter of 0.5cm (*Fig. 27B+C*). Every female constructs only one nest and can provide 12 to 18 brood cells with a fluid pollen-nectar mixture. The diurnal flight activity of *C. hederae* is strongly dependent on the availability of sunshine (BISCHOFF et al. 2005, own observations during collection in October 2012).

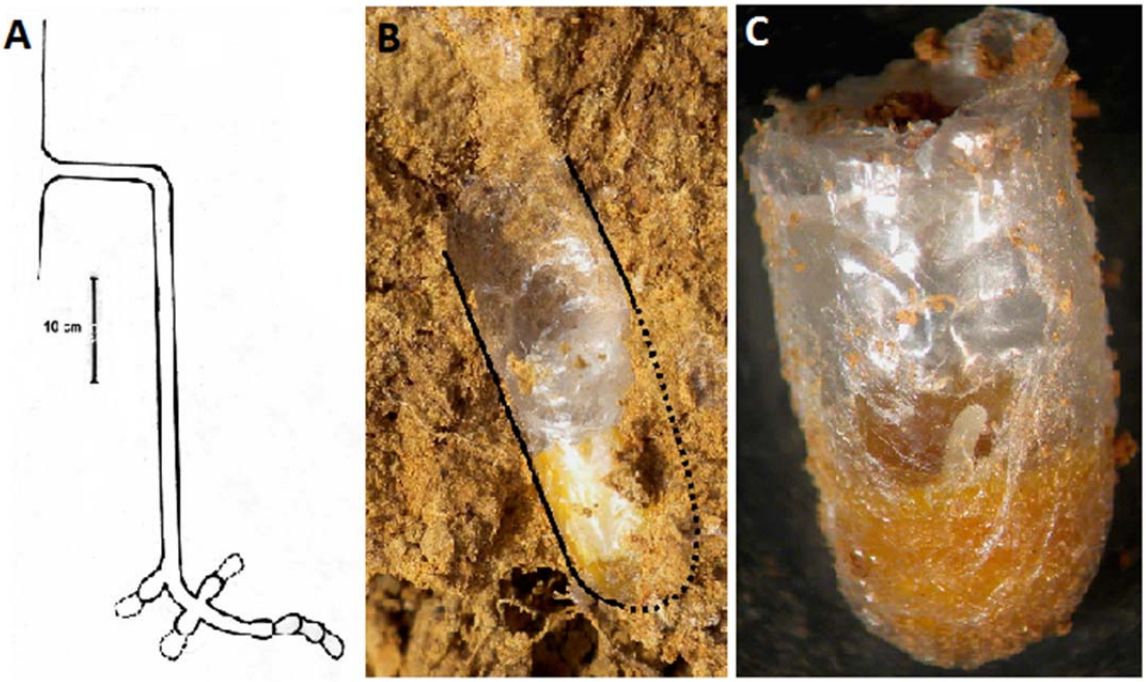

Figure 27. Nest of *Colletes hederae*. A: Nest architecture. From BISCHOFF et al. 2005. B: Partly excavated burrow which ends up in a brood cell (Photo: Karolin Schneider, Belgium, Brussels, Koekelberg, 2012). C: Brood cell with an egg inside (Photo: Karolin Schneider, collected in Belgium, Brussels, Koekelberg, 2012).

Colletes succinctus (Linnaeus 1758):

C. succinctus was already described by Linnaeus in 1758. An important characteristic to distinguish *C. succinctus* from its sibling species is its shiny galea which is matt in *C. halophilus* and *C. hederae* (SCHMIDT & WESTRICH 1993, KUHLMANN et al. 2007).

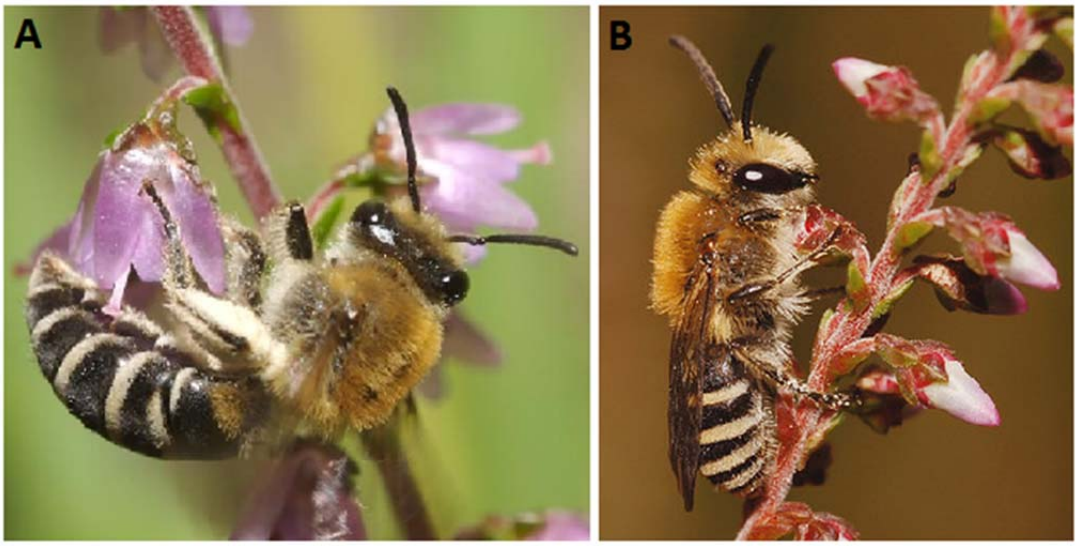

Figure 28. *Colletes succinctus* foraging on Ericaceae. A: Female with light yellow pollen loads on her hind legs. B: male. (Photos: Nicolas VEREECKEN).

C. succinctus was considered to be oligolectic on Ericaceae (i.e. *Calluna vulgaris*, *Erica* spp.; WESTRICH 1989). However females of *C. succinctus* are able to further collect minor amounts (<10% of pollen load) of pollen from the host plants of their sibling species *Hedera* and Asteraceae (Carduoidea, Cichorioideae, Asteroideae), as well as Apiaceae (MÜLLER & KUHLMANN 2008). Therefore *C. succinctus* is supposed to be polylectic, with strong preference on Ericaceae (>90% of pollen load according to MÜLLER & KUHLMANN 2008).

Figure 29. *Calluna vulgaris* (Ericaceae). Photo: Karolin Schneider, Belgium, Kalmthout, September 2012.

C. succinctus is widely distributed in Europe (*Fig. 30*). It occurs from north Britain to Greece in the south and from Portugal to Kazakhstan (KUHLMANN et al. 2007). The distribution of *C. succinctus* is consistent with the distribution of its host plants *Erica* spp. and *Calluna vulgaris* (*Fig. 30*).

Figure 30. Distribution of *Colletes succinctus*. Black dots show locations of *C. succinctus*. Shaded area indicates distribution of *Calluna vulgaris*. Doted area indicates distribution of *Erica* spp. From KUHLMANN et al. 2007.

The flight period of *C. succinctus* is from early July to the beginning of October (see *Fig. 18*; KUHLMANN et al. 2007). Compared to *C. halophilus* and *C. hederae* it is the earliest appearing species.

C. succinctus prefers lowlands or sand as nesting sites (KUHLMANN et al. 2007). As well as *C. halophilus* and *C. hederae* it nests in aggregations. The nest architecture is also similar to its sibling species. Each female (1) excavates several brood cells, (2) lines them with the polyester material which originates from Dufour's gland, (3) provides the cells with a pollen-nectar mixture, (4) deposits a single egg per cell, and (5) closes the cells with the cell lining material and a soil plug (*Fig. 31;* KUHLMANN et al. 2007).

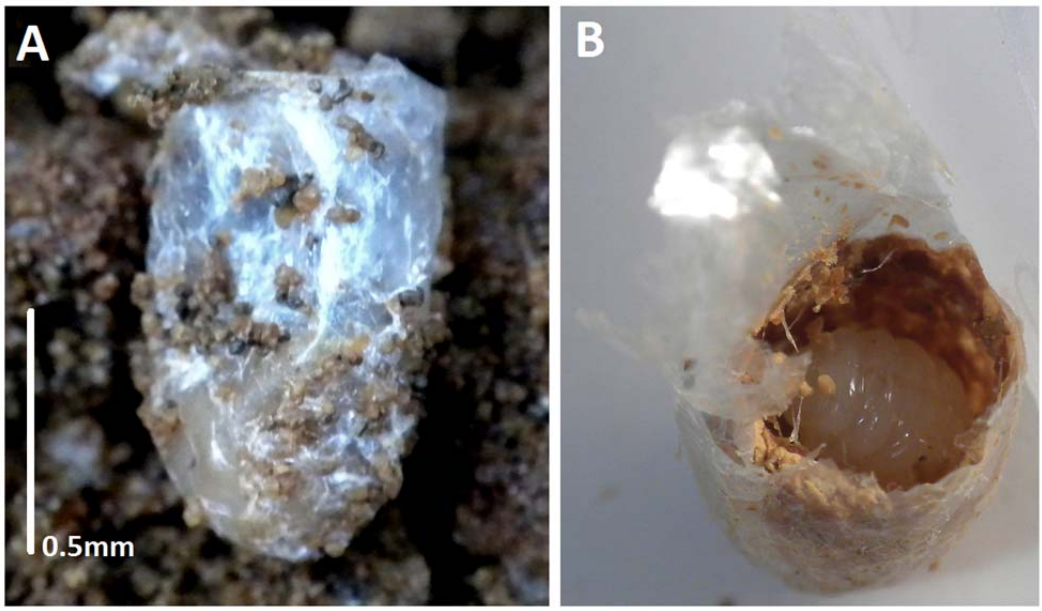

Figure 31. Brood cells of *Colletes succinctus*. A: Side view of excavated brood cell. Photo: Karolin Schneider, in November 2012, UK, Milford (Surrey). B: Top view of opened brood cell with large larva inside. Photo: Karolin Schneider, collected in November 2012, UK, Milford (Surrey).

3. Materials and Methods

3.1. Sampling

Three colletid bee species are included in this study: *C. halophilus*, *C. hederae* and *C. succinctus*.

Specimens and pollen loaves were collected in 2012 in Belgium, the Netherlands and England. Additional samples were included from the collection of the Laboratoire de Zoologie. The collecting locations and dates are listed in Table 1. Additional locations are visualized in Figures 32 to 35. According to the lifecycle of the three species, the material was collected from late August to early November.

Table 2. Collection locations and dates of the three colletid bee species.

Species	Samples	Number	Location	Date
Colletes halophilus	Males	11	Netherlands, Saefthinge 51°21'32.85"N 4°13'06.49"E	Sep 2008
		35		Sep 2012
	Females	32		Sep 2008
		11		Sep 2012
	Brood cells	117		Sep 2012
Colletes hederae	Males	51	Belgium, Brussels, Koekelberg (50°51'36.17"N 04°19'58.21"E)	Oct 2009
	Females	24		Sep 2012
		43	Belgium, Brussels, MuseumNSci (50°50'09"N 04°22'26"E)	Oct 2012
	Brood cells	96		Oct 2012
Colletes succinctus	Males	26	Belgium, Kalmthout 51°22'47.77"N 4°26'37.93"E	Aug 2010
		31		Sep 2012
	Females	6	United Kingdom, Surrey, Witley (51°9'5.915"N 0°39'46.951"W)	Sep 2010
		40		Sep 2012
	Brood cells	45	United Kingdom, Surrey, Milford (51°10'14.39"N 0°44'25.199"W)	Nov 2012

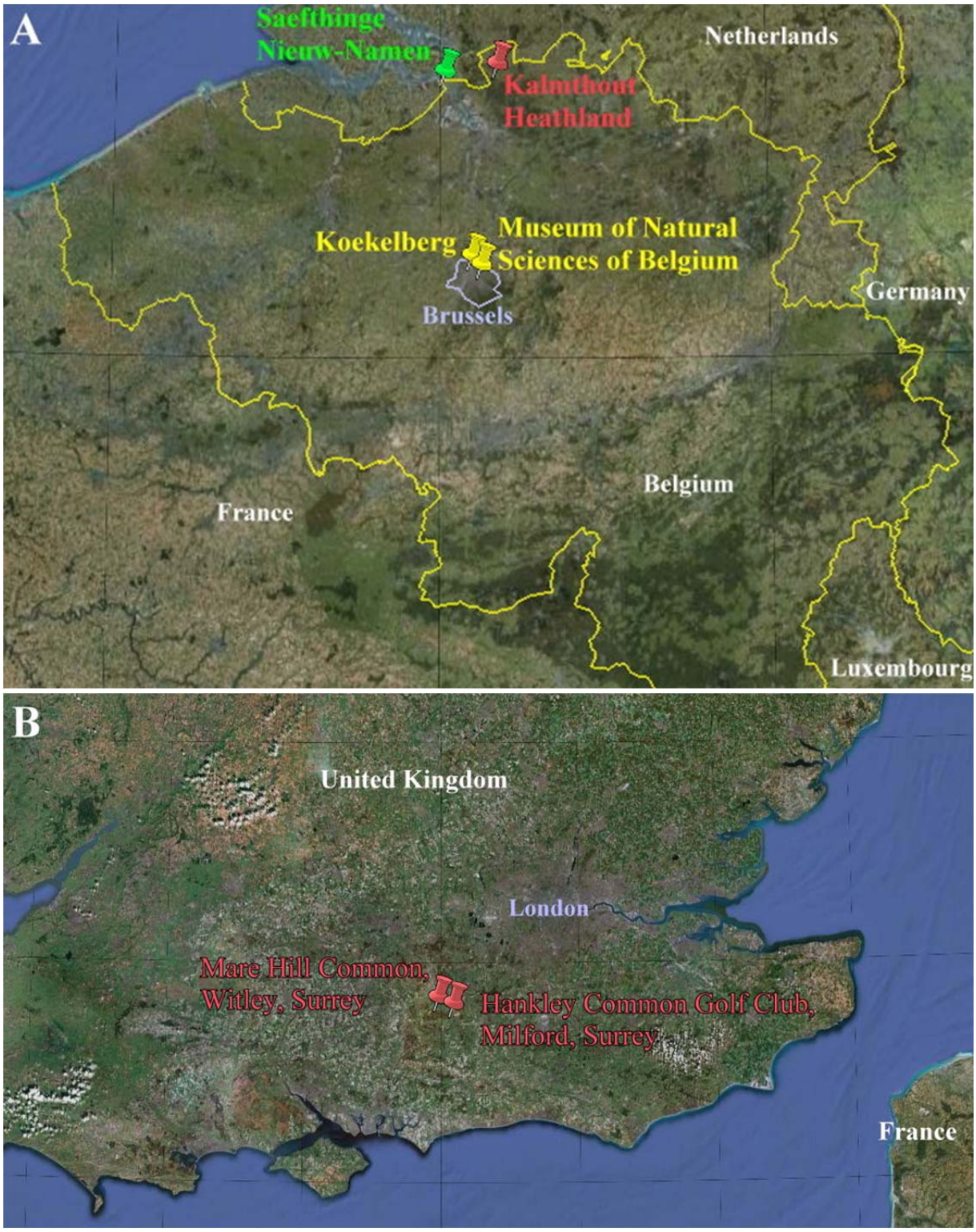

Figure 32. Collecting locations of colletid bees. A: Belgium and Netherlands. B: South England. Green: *Colletes halophilus*. Yellow: *Colletes hederae*. Red: *Colletes succinctus*.

Figure 33. Location of collection of *Colletes halophilus* (Netherlands, Saefthinge, Nieuw-Namen). A: Nesting site in sandy area. Nests are built in the dry sabulous ground. Photo: Karolin Schneider. B: Freshly excavated nest in an inclined wall. Red circle indicates the nest entrance. Photo: Karolin Schneider.

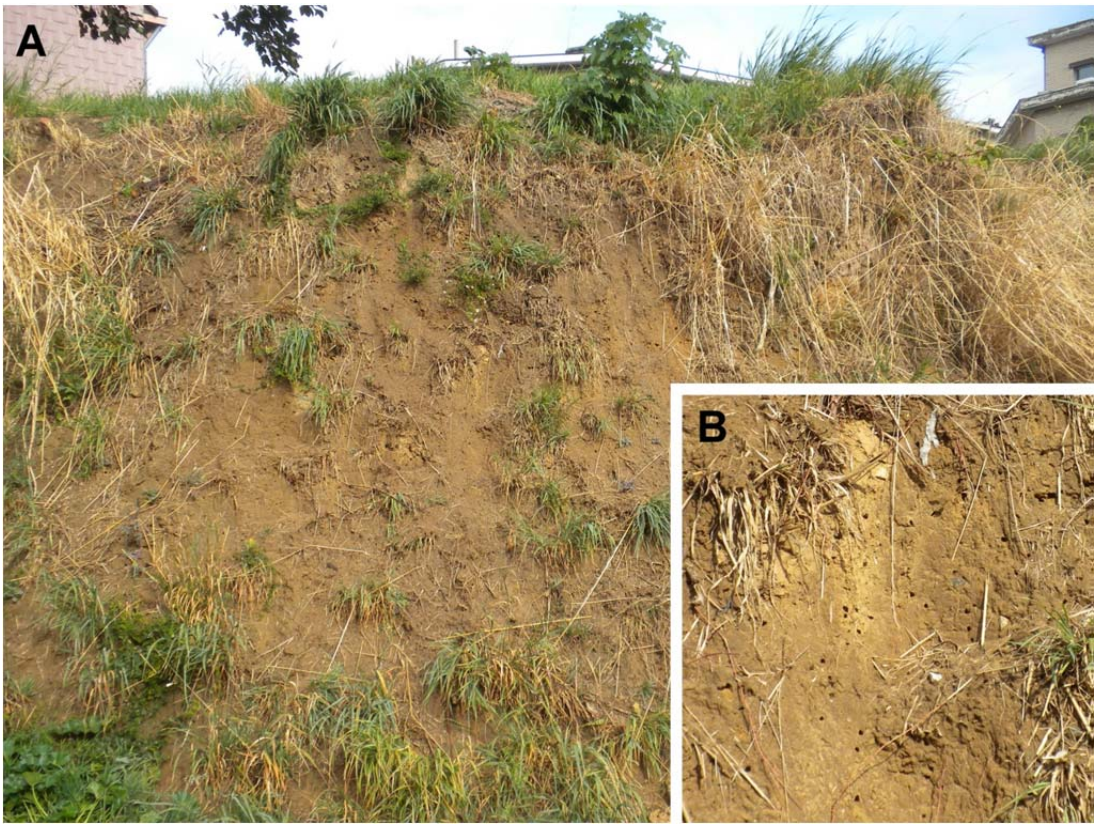

Figure 34. Location of collection of *Colletes hederae* (Belgium, Brussels, Koekelberg). A: Inclined slope covered with sparse vegetation. Photo: Karolin Schneider. B: Several nests are built in the stable soil. Photo: Karolin Schneider.

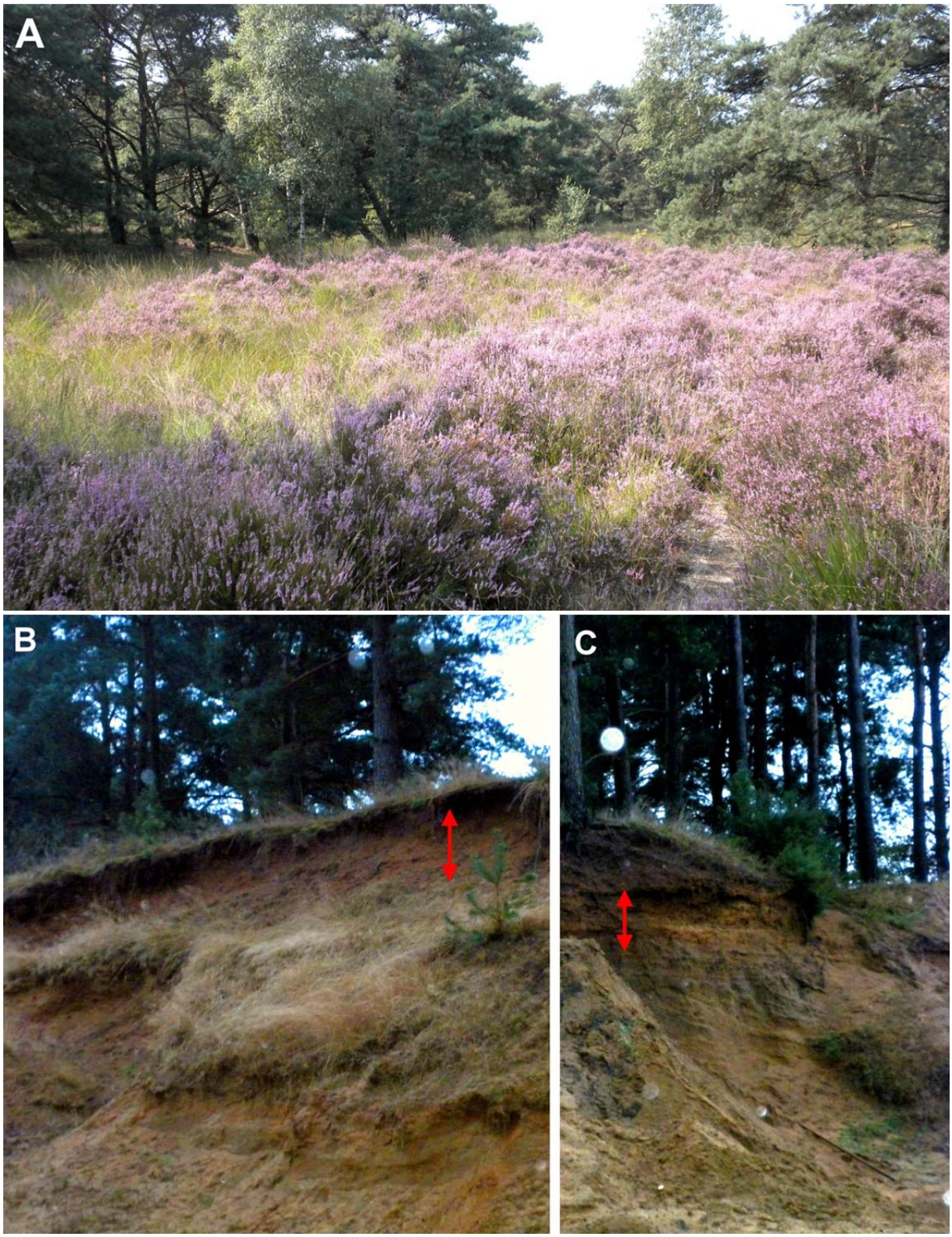

Figure 35. Collecting locations of *Colletes succinctus*. A: Broad dry habitat of host plant *Calluna vulgaris* where adult bees were collected (Belgium, Kalmthout Heathland). Photo: Karolin Schneider. B, C: Inclined slope with small vegetation where the brood cells were collected (United Kingdom, Surrey, Milford). Red arrows indicate the side of nests on the slope. Photos: Karolin Schneider.

Adult bees were collected during warm sunny days while flying above the nesting site or while foraging on their host plants. They were caught with an insect net and put into Eppendorf tubes individually. The Eppendorf tubes were labeled with a letter for the

collecting site and date plus a continuing number (e.g. A.1, B.2). Specimens were killed and stored in freezer of -20°C.

To collect brood cells, female bees were observed to find the entrances to their nests (see *Fig. 36*). The nests than could be excavated carefully with a shovel. Only finished (i.e. with yellowish pollen filled) brood cells were collected (*Fig. 37*), put into labeled Eppendorf tubes and stored in the fridge to avoid the death of the eggs and larvae.

Figure 36. Females of *Colletes halophilus* (Netherlands, Saefthinge) A+B: Females with pollen on their hind legs entering the nest entrance to fill the brood cells. C: Female bee digging a new nest. Photos: Karolin Schneider.

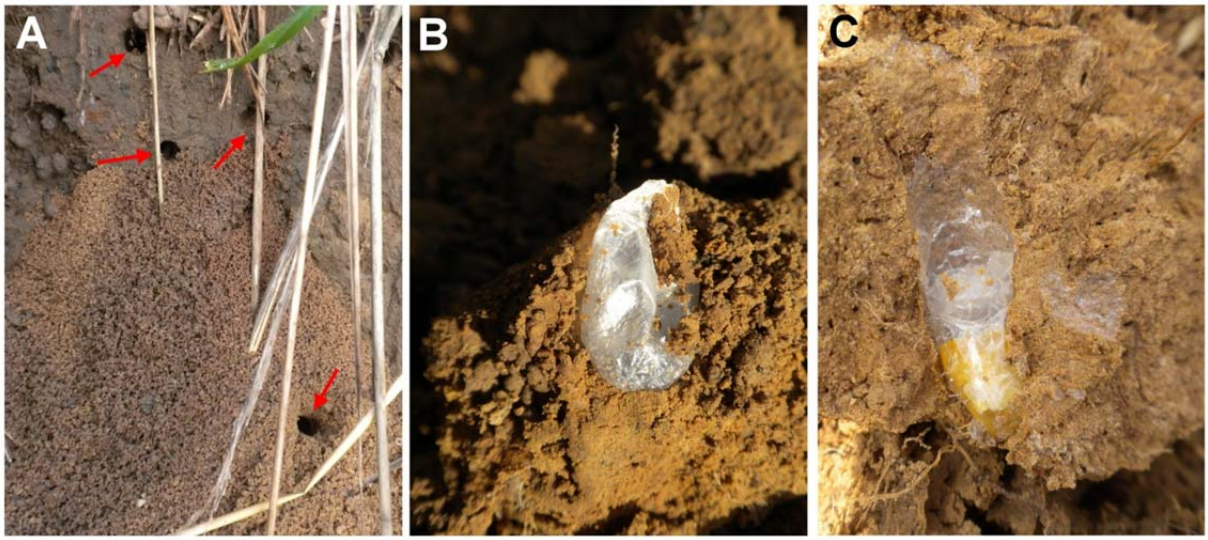

Figure 37. Nests and brood cells of *Colletes hederae*. A: Nest entrances. Photo: Karolin Schneider. B: Excavated brood cell without pollen provision. Not yet completed and closed by the female. Photo: Karolin Schneider. C: Excavated brood cell with yellowish pollen provision inside; already completed and closed by the female with the cell lining material. Photo: Karolin Schneider.

3.2. Analyses of adult bees

Stored frozen bees were defrosted for 30 minutes before they were weighed. Female bees that were covered with floral pollen were cleansed by compressed air. The fresh weight was measured by an analytical balance (Kern & Sohn GmbH, ABT220-4M) with a precision of 0.1mg. To determine the dry weight, the adult bees were lyophilized over night for 16 to 18 hours. They were weighed immediately and re-stored in a freezer of -20°C.

To estimate the size of the bees, the right anterior wings were pulled out with tweezers and arranged on millimeter paper (*Fig. 38*). Pictures were taken with the aid of an Olympus stereo microscope (magnification 1.5 times) and a Nikon D70 camera (focal distance F=40). To save the wings they were separately put into little plastic bags and labeled with the sample numbers. The photographs were converted from jpg to tps file format using the software package tpsUtil (F. J. ROHLF). The distance between landmarks 1 and 3 (see *Fig. 38*) was measured with the software tpsDig2 (F. J. ROHLF). It is proportional to the body-length of the bee (BOSCH & VICENS 2002).

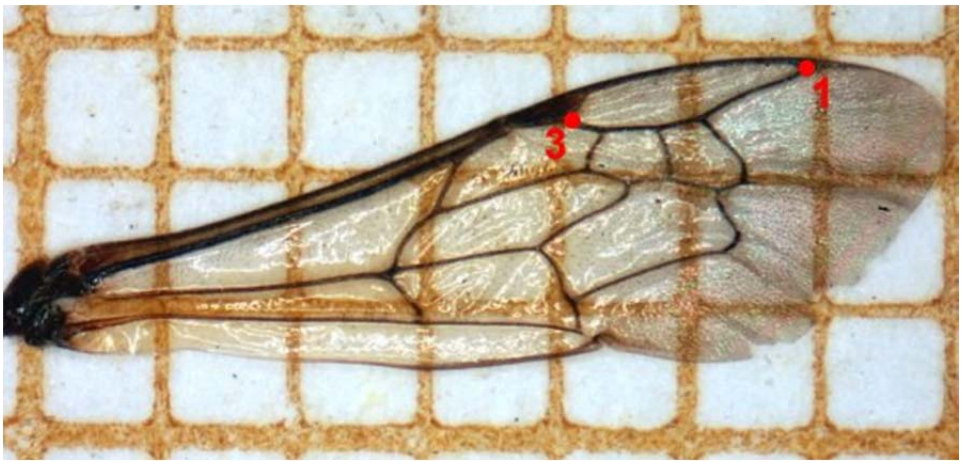

Figure 38. Right anterior wing of male bee of *Colletes succinctus*. Arranged on millimeter paper, photo taken under a stereo microscope.

3.3. Analysis of brood cells

Soil and sand grains were removed from the collected brood cells with the aid of a brush under a stereo microscope. The cleansed brood cells were put into new Eppendorf tubes and stored in the fridge. To separate the egg or larva from the pollen loaf, the brood cell was cut open. The egg or larva was removed with tweezers and put into a new Eppendorf tube labeled with the same inscription. After removing the eggs or larvae, the fresh weight of the pollen loaves was measured by an analytical balance with a precision of 0.1mg. Subsequently the pollen loaves were lyophilized and the dry weight was measured. The larvae of *Colletes succinctus* were already very large. That is why the entire brood cells with the containing larvae were weighed freshly.

The living eggs and larvae which were transferred to new labeled Eppendorf tubes were directly put into a deep-freezer of -80°C to avoid the destruction of the cells and DNA before the analysis.

3.3.1. Sex determination of eggs and larvae

To determine the sex of the eggs or larvae the method of flow cytometry was used. Before analyzing the samples they had to be treated with a DNA coloration solvent. The quantity of the colored DNA than can be detected by the flow cytometer.

Bee females are diploid (e.g. WCISLO 2000, MICHENER 2007) and have therefore twice as much DNA in their cell nucleus than male bees that are haploid. With the aid of the flow cytometer both sexes can be distinguished due to the different coloration signals (strong signal for diploid/female cells and weaker signal for haploid/male cells).

The eggs or small larvae were used totally for the analysis. The large larvae were cut into two pieces and only one half was used for the analysis.

The majority of the samples were prepared by a one-step solution (Cystein UV Ploidy 1 step solution – order n°05-5001). The samples were comminuted in 200µl of a coloration solution (DAPI). Then 800µl of the same colorant were added and the samples were directly ready for the analysis. Ten samples were prepared by a two-step method (Cystein DNA 2 step solution – order n°05-5005) to see which method will be the best. First they were comminuted in 200µl extraction buffer. Than 800µl of a staining solution was added and after 2 minutes the samples were ready for the analysis. There were no striking differences between the two methods. Therefore the faster one (DAPI) was used for all other samples. The prepared samples were placed into special tubes for the cytometry. They were analyzed with a Partec© flow cytometer. The cytometer was calibrated with the heads of a male and a female bee of *Colletes hederae*. The adult bees were prepared by the one-step solution (1ml of DAPI).

ROOIJAKKERS & SOMMEIJER (2009) determined the larvae of *Colletes halophilus* by observing the genital appendages of the larvae with a stereoscopic microscope. This method was not useful for the present study due to the loss of pollen loaf caused by large larvae.

3.3.2. Polypeptide quantification of pollen loaves

For each species, six lyophilized brood cells (3 pollen loaves with male eggs/larvae and 2 or 3 with female eggs/larvae) were analyzed. At first the cell lining was removed with tweezers under a stereo microscope (Wild Heerbrugg, Van Hopplynus instruments). The sticky pollen-nectar mixture was mashed by a scalpel to ensure homogeneity. Per pollen loaf 3 samples were made. For each sample about 5 mg of the pollen loaf is necessary (see Vanderplanck et al., submitted). To take into account the loss of weight because of lyophilization, about 7 mg of the homogenized loaf was weighed in the Eppendorf tubes. Afterwards the samples were

lyophilized again, to remove newly absorbed humidity. The exact dry weight of the samples was noted (see *Tab. 3*).

Table 3. Exact dry weight of the samples of pollen loaves used for protein quantification. For each species three pollen loaves of male eggs/larvae and two to three pollen loaves of female eggs/larvae were considered for the analysis. Three samples were taken of each pollen loaf.

Colletes halophilus			*Colletes hederae*			*Colletes succinctus*		
Pollen Loaves	Sample	Weight (mg)	Pollen Loaves	Sample	Weight (mg)	Pollen Loaves	Sample	Weight (mg)
female	1	8.33	**female**	1	6.71	**female**	1	6.14
	2	8.36		2	6.51		2	6.13
	3	8.43		3	6.65		3	6.25
female	1	7.74	**female**	1	8.81	**female**	1	6.65
	2	7.22		2	8.76		2	6.66
	3	6.50		3	8.90		3	5.56
female	1	8.49	**male**	1	6.48	**female**	1	7.15
	2	8.48		2	6.58		2	7.24
	3	8.86		3	6.32		3	7.27
male	1	7.79	**male**	1	6.00	**male**	1	5.94
	2	7.79		2	6.28		2	5.63
	3	7.74		3	6.12		3	5.46
male	1	7.04	**male**	1	7.17	**male**	1	6.78
	2	6.89		2	7.09		2	6.84
	3	6.51		3	7.29		3	6.86
male	1	6.58				**male**	1	6.60
	2	5.97					2	6.60
	3	5.94					3	6.56

3.3.2.1. Polypeptide extraction

Due to the contained sugar of the nectar, the samples became very hard after lyophilization. The first step of the polypeptide quantification was to degrade the solid pollen nuggets and break open the pollen grains. Therefore an extraction buffer was added and the tubes were heated in a water bath of 90°C for 5 to 20 minutes. The extraction of the polypeptides from the solution was carried out with 500µl phenol. 400µl of the 500µl phenol which contained the total extracted polypeptides of the sample were used for the protein quantification (see *Fig. 39*). The loss of 100 µl phenol-polypeptide solution was considered in the calculation of the polypeptide concentration.

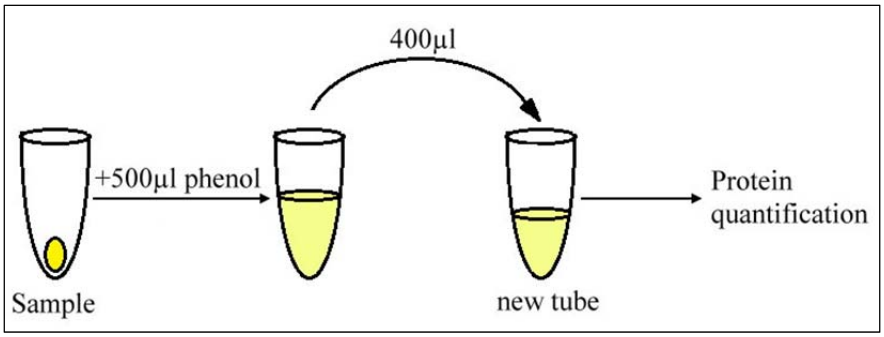

Figure 39. Scheme showing the extraction with phenol. 500µl of phenol were added to the pollen sample. Only 400µl of the phenol containing the total extracted polypeptides were transferred to a new tube and utilized for the polypeptide quantification.

To precipitate the extracted polypeptides they were incubated with ammonium acetate (in 100% methanol) overnight. To avoid contamination (lipids, pigments, cell material) the solutions were purified twice with methanol 100% and once with acetone 80%. The purified polypeptides were resolved in 500µl guanidine-hydrochloride-buffer (*Fig. 40*).

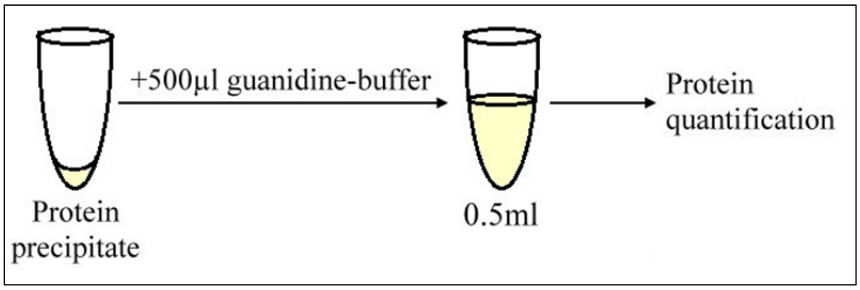

Figure 40. Scheme showing the dilution of the extracted and purified polypeptides with guanidine-hydrochloride-buffer. The protein content was measured for the 0.5ml polypeptide solution.

3.3.2.2. Polypeptide quantification

The quantification of the polypeptides was made with a BCA Protein Assay Kit (Pierce, Thermo Scientific): a coloration solvent was used to detect the polypeptide contents. The calibration curve was created with the aid of different concentrations of BSA in the guanidine-buffer. To measure the absorption, the samples were placed in the wells of a microwell plate as triplicates (see *Fig. 41* and *42*). The absorption of the samples was measured at a wavelength of 570 nm at three different time intervals: t0 (immediately after incubation), t5 (after five minutes) and t10 (after 10 minutes). The measurement was done by a "FLUOstarOPTIMA" (BMG Labtech) apparatus and the software "OPTIMA" (BMG Labtech). (For details for the polypeptide quantification see *Appendix 1*.)

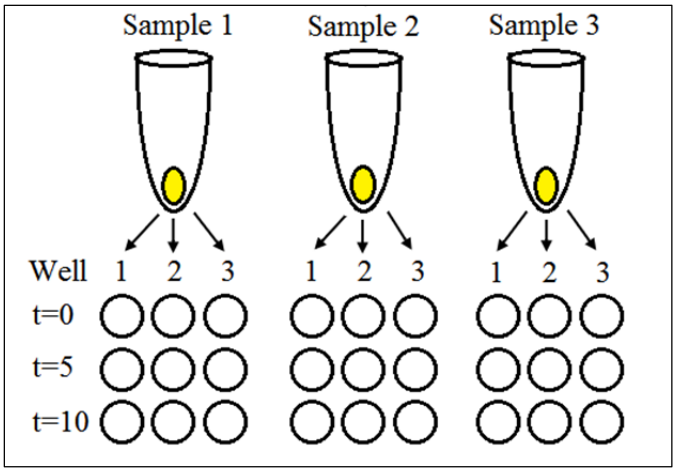

Figure 41. Analysis of one pollen loaf. The extracted and purified polypeptides of the 3 subsamples was filled in 3 wells of the microwell plate. The absorption was measured at three different time intervals (t0, t5, t10).

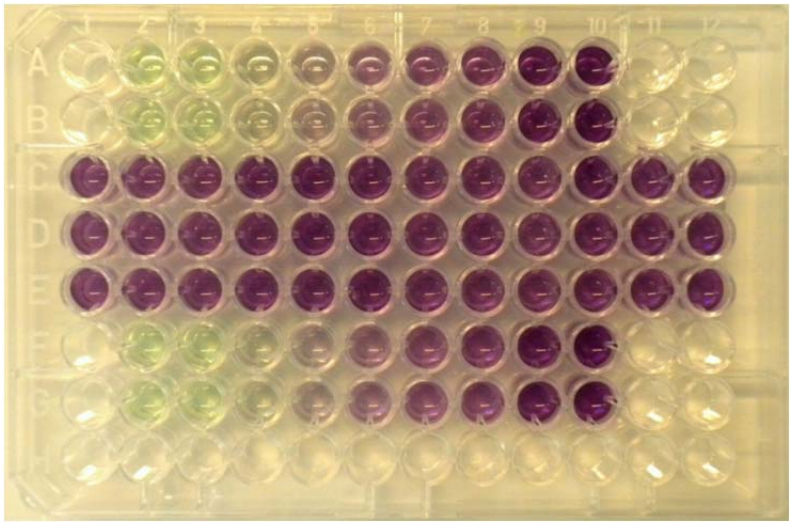

Figure 42. Microwell plate for analysis of protein quantity. In the wells of lines A, B, F, and G the standard for the calibration curve was applied. It starts with the blank and ends up in the highest concentration. In the wells of lines C, D, and E the samples were applied. Green coloration: low protein level. Purple coloration indicates high protein level.

3.3.2.3. Calculations

The raw data was interpreted by the software "OPTIMA-Data Analysis" (BMG Labtech) and displayed as unit µg/ml. The calculations to achieve the protein content per mg of the pollen loaves were carried out with Microsoft Excel 2010 and are presented in the following. The different steps are visualized in Figure 43.

To calculate the protein content per mg of the whole pollen loaf, the mean values of the triplicates, repeats and samples has to be calculated. The absorption was measured at three different time intervals (t0, t5, t10). The mean value of these 3 time intervals was calculated (*Fig. 43*C). This mean was divided by the amount (mg) of the used sample to calculate the protein content (µg) per mg pollen loaf given by each single well (*Fig. 43*D). Of each sample a triplicate analysis was made in the microwell plate (well 1, well 2, well 3). The mean value

between these three wells was calculated (*Fig. 43*E). For each pollen loaf 3 samples were analyzed. The mean protein content of the three samples was calculated (*Fig. 43*F). The final result shows the protein content in µg per mg of the pollen loaf.

To calculate the relative protein content of one pollen loaf, the protein content per mg has to be divided by 10, due to the fact that 1µg protein per 1mg pollen loaf is equivalent to 1/1000 or 0.1% respectively.

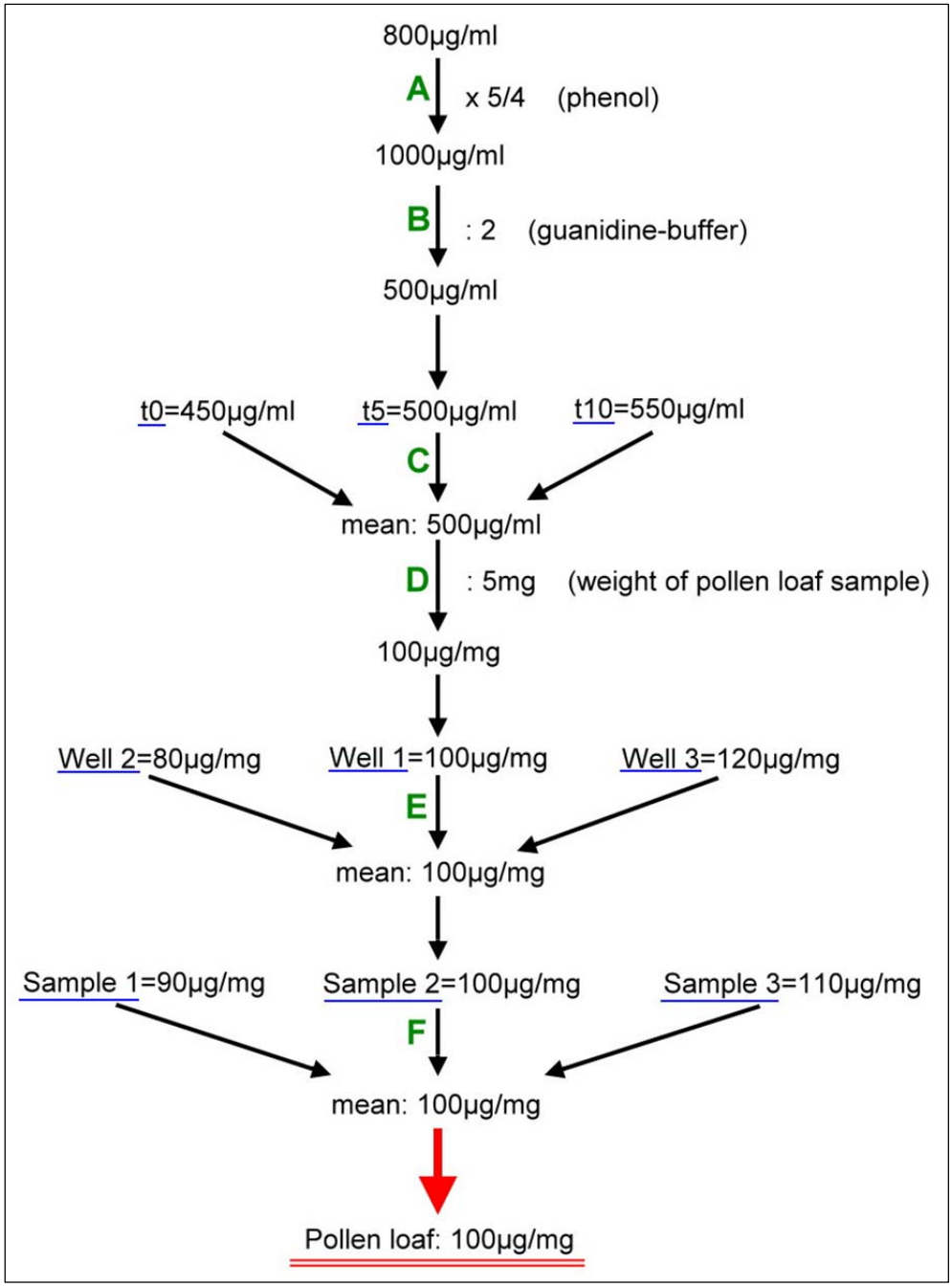

Figure 43. Scheme of calculation of protein content in µg per mg of the pollen loaf. An example with intended values is shown. The first value represents the result calculated by the software. A: Multiplication by 5/4. B: Division by 2. C: Mean of the values of all three measured time intervals. D: Division by the weight of the pollen sample. E: Mean of the values of all three wells (triplicate analysis). F: Mean of the values of all three samples taken of one pollen loaf.

3.4. Statistical analysis

The tables and diagrams to visualize the results, mean values and standard deviations were made by Microsoft Excel 2010 (Microsoft Corporation).

Statistical analyses were carried out with the statistical software "R" version 2.15.1 and 2.15.2 respectively (CHAMBERS et al., Bell Laboratories, Lucent Technologies). The normality of distribution was checked with the Shapiro-Wilk normality test (SHAPIRO & WILK 1965). To test the homogeneity of variances the Bartlett-test (BARTLETT 1937) was used. To detect significant differences an analysis of variance (ANOVA) and the Tukey-HSD-test (TUKEY J.W.; multiple comparisons of means, honestly significant difference) were conducted. At first, the real values of the results were used for the tests. When there was no normal distribution or the variances not homogeneous, the statistical tests were made again with the logarithm of the values. To compare interspecific and intraspecific differences, box plot diagrams were drawn with the aid of the same software.

4. Results

4.1. Size of adult bees

The wings of 308 adult individuals were measured (*Tab. 4*, *Fig. 44,45*).

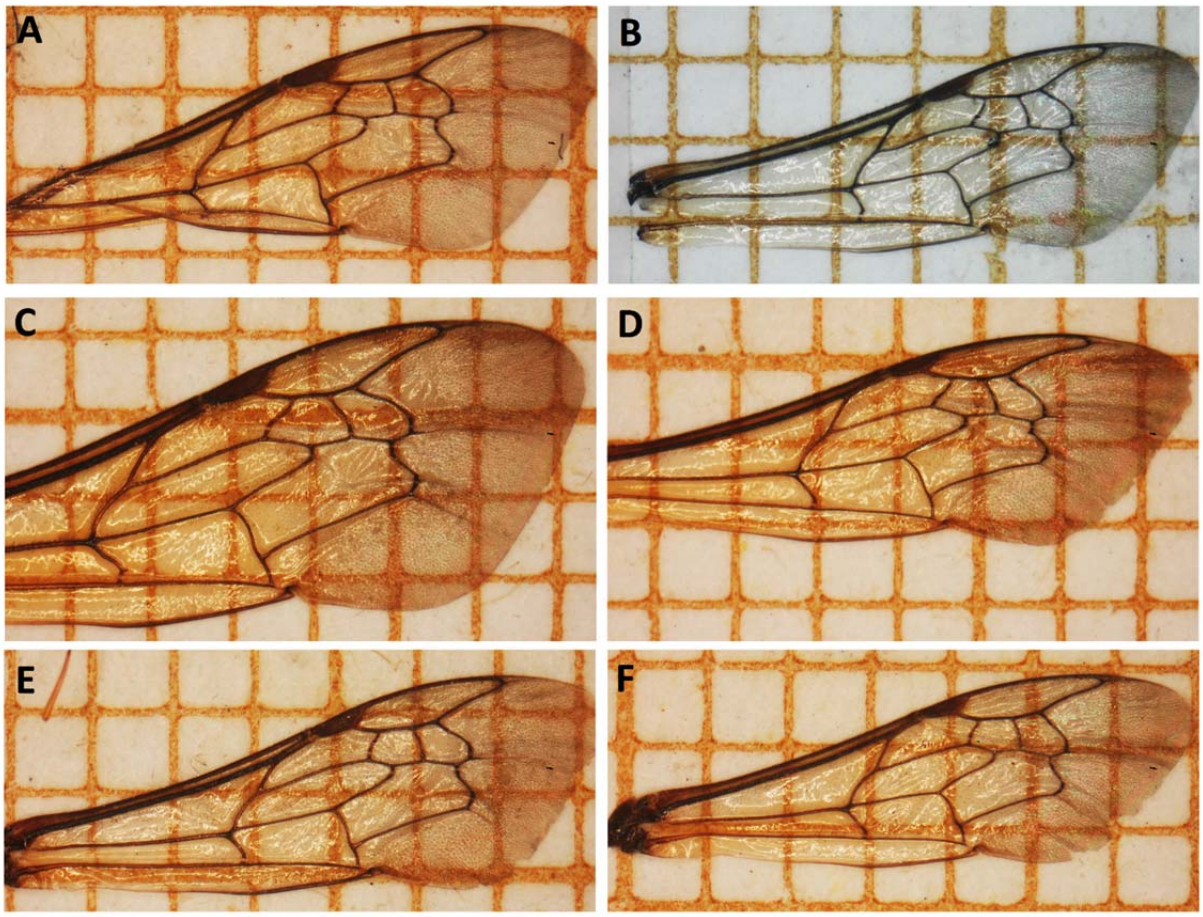

Figure 44. Right anterior wings of adult bees. A: *C. halophilus*, female. B: *C. halophilus*, male. C: *C. hederae*, female. D: *C. hederae*, male. E: *C. succinctus*, female. D: *C. succinctus*, male. Photos: Karolin Schneider.

Table 4. Wing size of the three colletid bee species. The table shows the distance of the landmarks 1 and 3 of the adult wings. Column 4 to 7: all values are given in mm.

Species	Sex	Number	Mean	SD	Smallest distance	Largest distance
Colletes halophilus	female	n=45	2.16	0.099	1.90	2.41
	male	n=42	1.74	0.057	1.63	1.91
Colletes hederae	female	n=51	2.44	0.094	2.26	2.65
	male	n=67	2.07	0.073	1.90	2.22
Colletes succinctus	female	n=57	1.86	0.082	1.64	2.01
	male	n=46	1.64	0.066	1.53	1.80

Colletes halophilus

The smallest distance between landmarks 1 and 3 on the wings of the 45 considered *C. halophilus* females is 1.90mm and the largest distance 2.41mm (*Fig. 45*). On average the distance is 2.16mm. For the 42 male adults the smallest distance is 1.63mm and the largest

distance is 1.91mm. The mean distance is 1.74mm. The standard deviation for the female and male bees is 0.099mm and 0.057mm respectively (*Tab. 4*).

Colletes hederae

The smallest distance measured for the 51 females of *C. hederae* is 2.26mm and the largest distance is 2.65mm (*Fig. 45*). The mean distance of landmarks 1 and 3 is 2.44mm; with a standard deviation of 0.094mm. The largest of the 67 male wings has a distance between landmarks 1 and 3 of 2.22mm and the smallest 1.90mm. On average the distance on the male wings is 2.07mm. The standard deviation is 0.073mm (*Tab. 4*).

Colletes succinctus

The smallest distance of the distance of landmarks 1 and 3 of the 57 females of *C. succinctus* is 1.64mm and the largest distance is 2.01mm (*Fig. 45*). The mean is 1.86mm and the standard deviation is 0.082mm. The smallest distance measured for the 46 males is 1.53mm and the largest is 1.80mm. On average the distance between landmarks 1 and 3 is at 1.64mm; with a standard deviation of 0.066mm (*Tab. 4*).

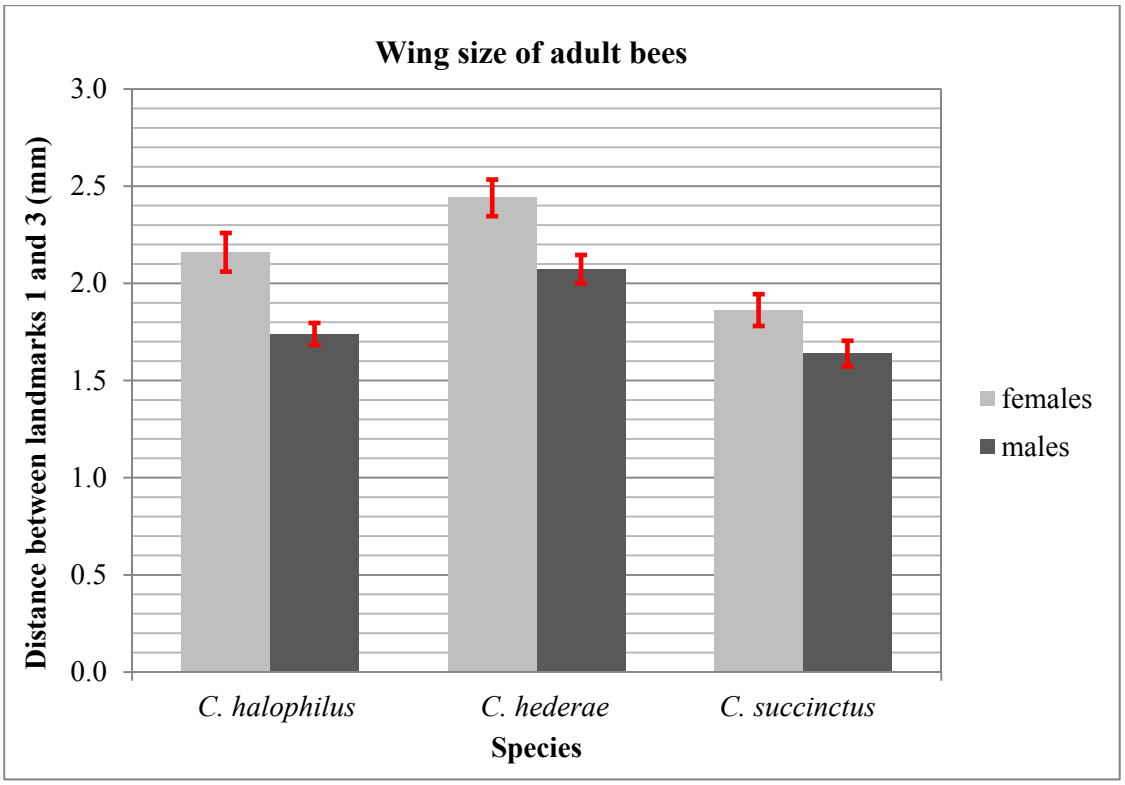

Figure 45. Wing size of the adult bees. The mean value of the distance between landmarks 1 and 3 for each species and sex is illustrated by bars. The standard deviation is marked by red indicators.

Intraspecific variability

The resulting values for the wing size are normally distributed. Homogeneity of variances could not be proved.

Due to the fact that the distance between the landmarks 1 and 3 on the wings of the bees corresponds to their total body size, the wing size is used for the relative body size of the bees.

Within all species the female individuals are larger than their male counterparts. The difference between the body size of males and females is significant (*Fig. 46*).

The differences in relative size between males and females are illustrated in Figure 47. The sexual dimorphism is the largest in *C. halophilus*. The males are on average 19.5% smaller than the female bees. The female bees of *C. hederae* are on average about 15% larger than the male individuals. For the adults of *C. succinctus* the difference between the size of male and female bees is 12%.

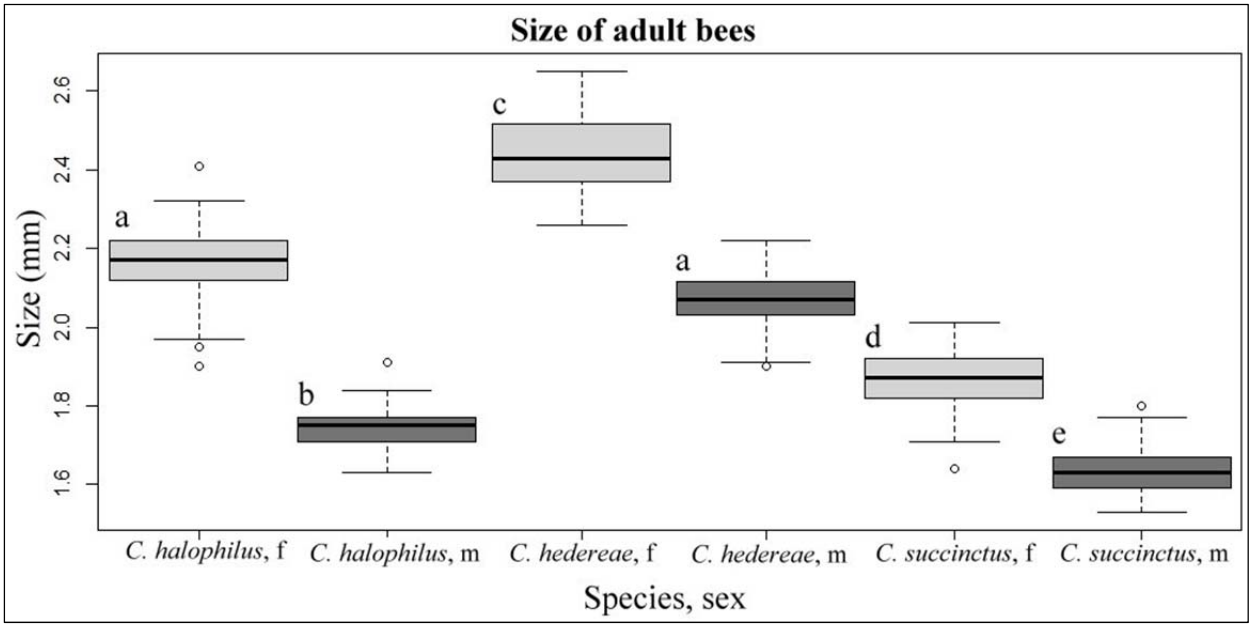

Figure 46. Boxplot diagram showing differences in the relative size of adult bees. Light grey: female bees. Dark grey: male bees. The results which differ significantly from each other are labeled with different small letters.

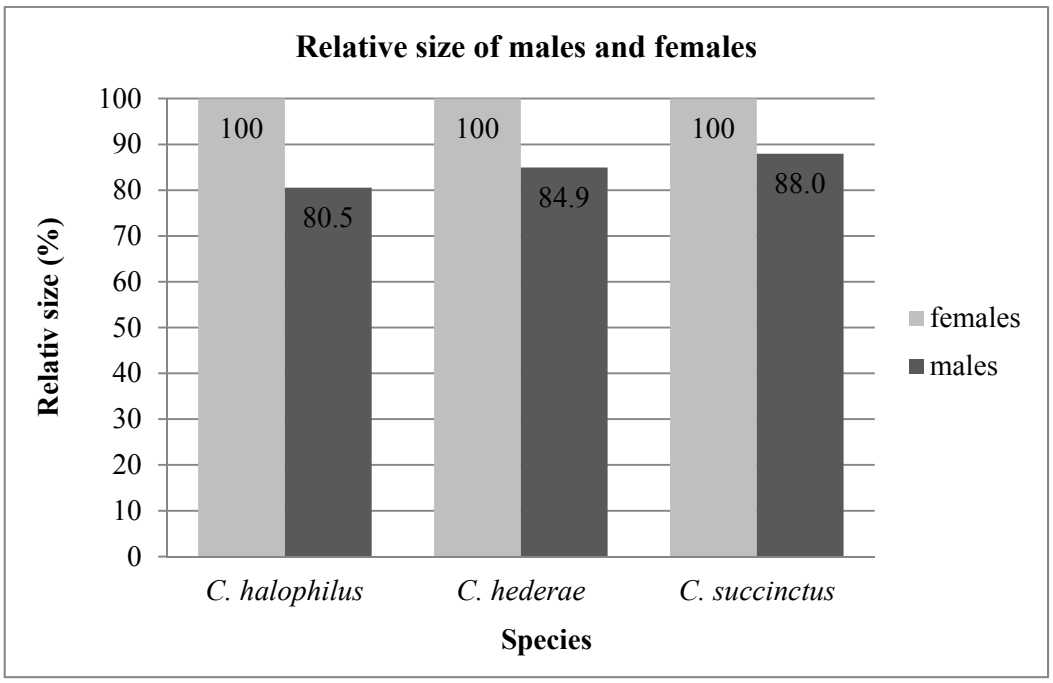

Figure 47. Ratio between the size of female and male bees. For each species the size of the females is taken as 100% to compare the relative size of both sexes within a species.

Interspecific variability

In general, the sizes within the sexes of the three species are significant (see *Fig. 46*). There is no significant difference between the females of *C. halophilus* and the males of *C. hederae*.

The relative body size of females and males is illustrated in Figure 48. *C. hederae* is the largest species and *C. succinctus* is the smallest species. The females of *C. hederae* are on average 11.5% larger than those of *C. halophilus* and even 24% larger than the females of *C. succinctus*. The males of *C. halophilus* and *C. succinctus* are 16% and 21% respectively, smaller than the males of *C. hederae*.

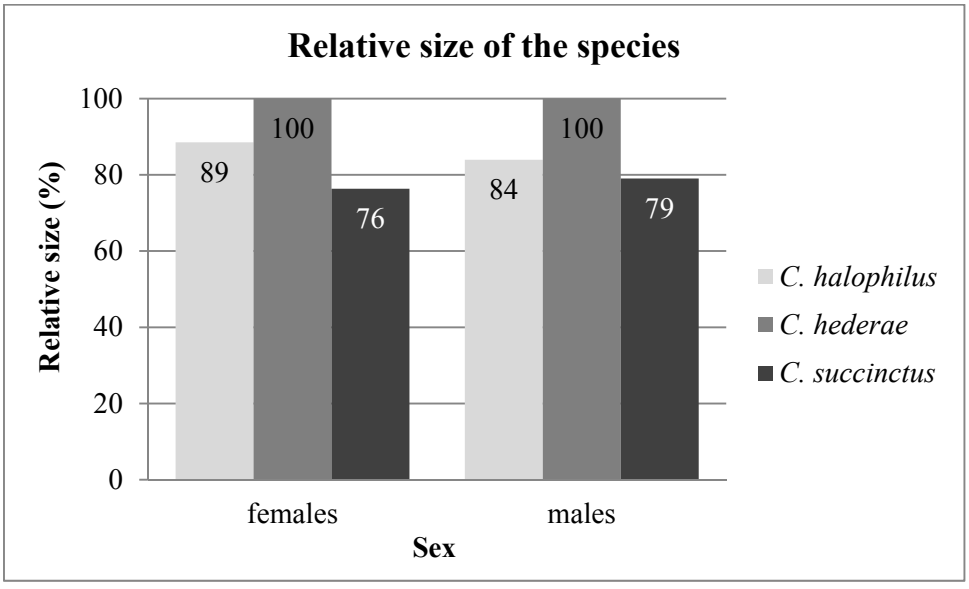

Figure 48. Ratio between the sizes of the three bee species. For the largest species *C. hederae* the body size is taken as 100% to compare the relative size of the three species.

4.2. Weight of adult bees

The fresh and dry weight of 310 adult individuals was measured (*Tab. 5*, *Fig. 49*).

Table 5. Weight of the three colletid bee species. The table shows the dry weight of the adult bees. Column 4 to 7: all values are given in mg.

Species	**Sex**	**Number**	**Mean**	**SD**	**Lowest weight**	**Highest weight**
Colletes halophilus	female	n=46	37.3	8.02	23.9	59.9
	male	n=43	16.0	2.23	10.8	22.2
Colletes hederae	female	n=51	42.2	9.78	24.6	66.8
	male	n=67	16.4	2.07	10.4	22.6
Colletes succinctus	female	n=57	23.5	4.96	12.9	36.6
	male	n=46	11.0	2.25	8.0	20.2

Colletes halophilus

The measured values of all females of *C. halophilus* range from 23.9mg to 59.9mg (*Tab. 5*). The majority of results are in the range between 30mg and 50mg. The mean dry weight of the females is 37.3mg and the standard deviation is 8.02mg (*Fig. 49*). The majority of results for the males are in the range between 10mg and 20mg. The lowest weight is 10.8mg and the highest weight is 22.6mg. On average the males weigh 16mg. The standard deviation amounts to 2.23mg.

Colletes hederae

The majority of values of the weight of females of *C. hederae* range from 30mg to 60mg. The lowest weight measured for the females is 24.6mg and the highest weight is 66.8mg (*Tab. 5*). On average the females weigh 42.2mg (*Fig. 49*). The standard deviation is 9.78mg. The heaviest males weigh 22.6mg and the slightest males weigh 10.4mg. The majority of males are in the range between 10mg and 20mg. The mean weight of the males is 16.4mg; with a standard deviation of 2.07mg.

Colletes succinctus

The results for the female bees of *C. succinctus* range from 12.9mg to 36.6mg. The majority of values are in the range between 15mg and 30mg (*Tab. 5*). On average the females weigh 23.5mg and the standard deviation is 4.96mg (*Fig. 49*). The results of the males range around 10mg. The lowest weight is 8mg and the highest weight is 20.2mg. With a standard deviation of 2.25mg the mean value is 11mg.

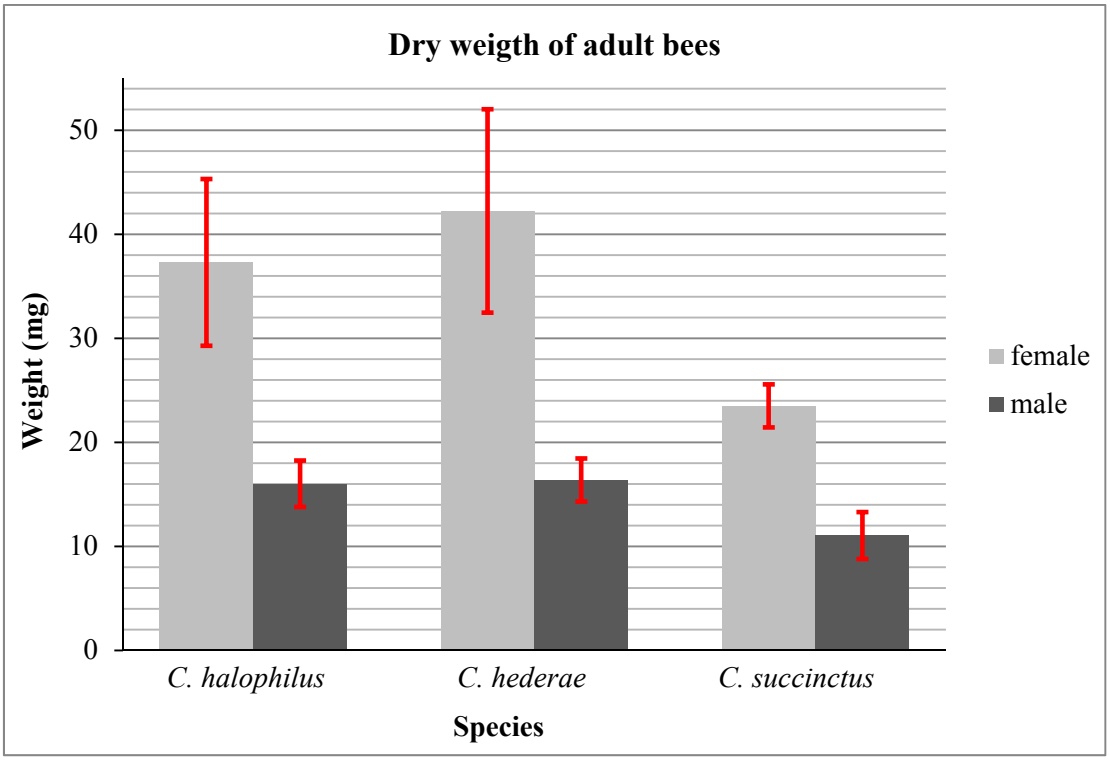

Figure 49. Dry weight of the adult bees. The mean value of the dry weight for each species and sex is illustrated by bars. The standard deviation is marked by red indicators.

Intraspecific variability

The resulting values for the dry weight are normally distributed. Homogeneity of variances could be proved for the logarithm of the values. Further statistical analyses were all carried out with the logarithm of the measuring results.

Within all species the female individuals are heavier than the male individuals. The difference between the logarithm of the female and the male weight is significant (*Fig. 50*).

The difference in dry weight between males and females is illustrated in Figure 51. The largest difference between males and females was found in *C. hederae*. The weight of the males is on average 61% smaller than the weight of the females. The female bees of *C. halophilus* are on average 57% heavier than the male individuals. The difference between the sexes is the smallest in *C. succinctus*. The males are on average only 53% lighter than the female bees.

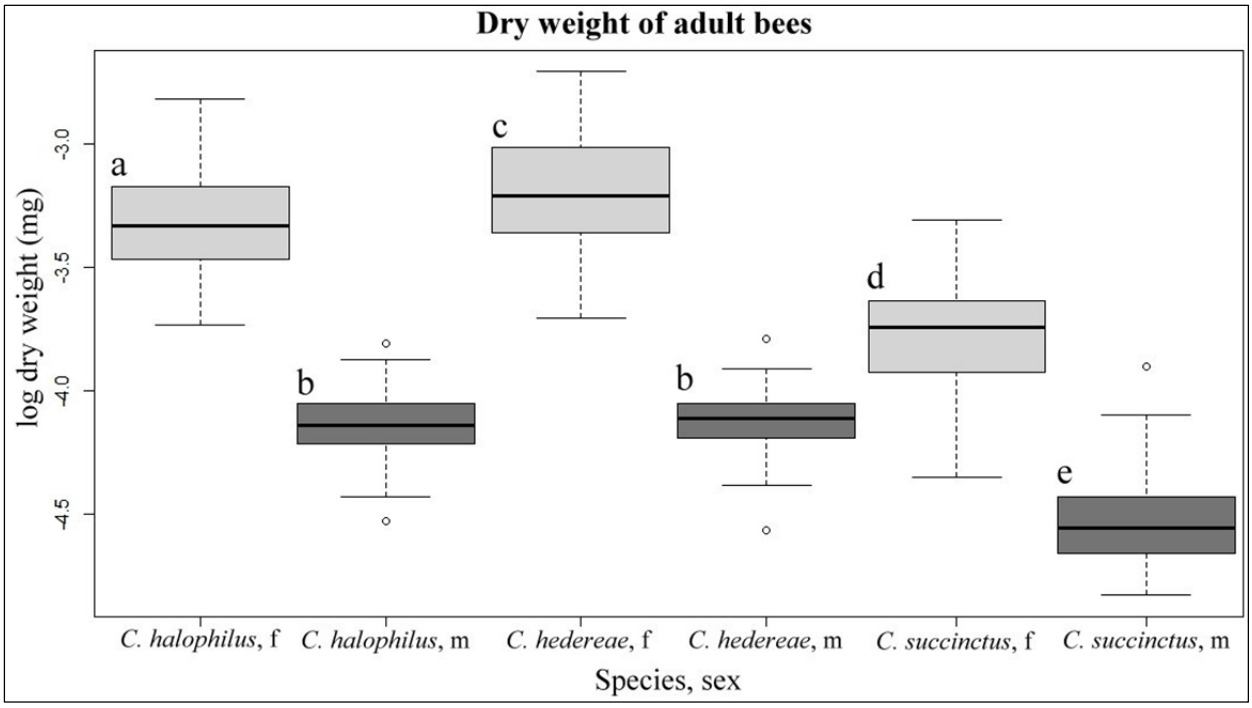

Figure 50. Boxplot diagram showing differences in the dry weight of adult bees. Light grey: female bees. Dark grey: male bees. The results which differ significantly from each other are labeled with different small letters.

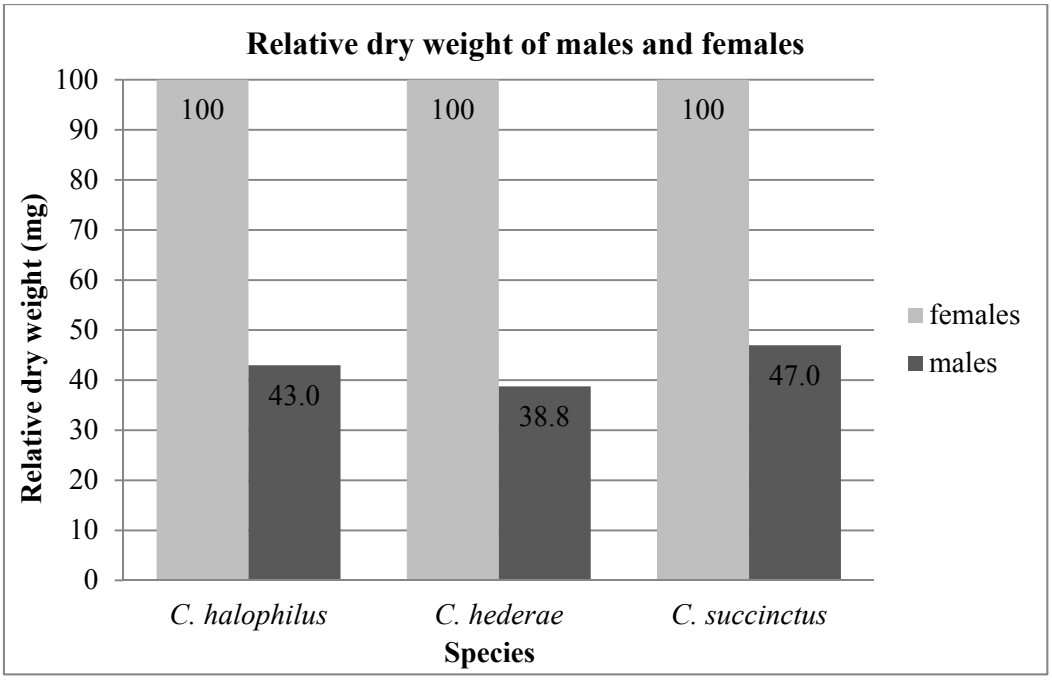

Figure 51. Ratio between the weight of male and female bees. For each species the dry weight of the females is taken as 100% to compare the relative weight of both sexes within a species.

Interspecific variability

The differences in the logarithm of the dry weight of females and males are significant between the three species (*Fig. 50*). However the difference between females of *C. halophilus*

and *C. hederae* is less significant than between the other species. There is only no significant difference between the males of *C. halophilus* (16mg) and the males of *C. hederae* (16,4mg).

The results show that *C. hederae* is the heaviest species, followed by *C. halophilus*. *C. succinctus* is the lightest species (*Fig. 52*). The females of *C. hederae* are on average 12% heavier than those of *C. halophilus* and 44% heavier than the females of *C. succinctus*. The males of *C. halophilus* are only 2% lighter than the males of *C. hederae*. The males of *C. succinctus* are 33% lighter than the males of *C. hederae*.

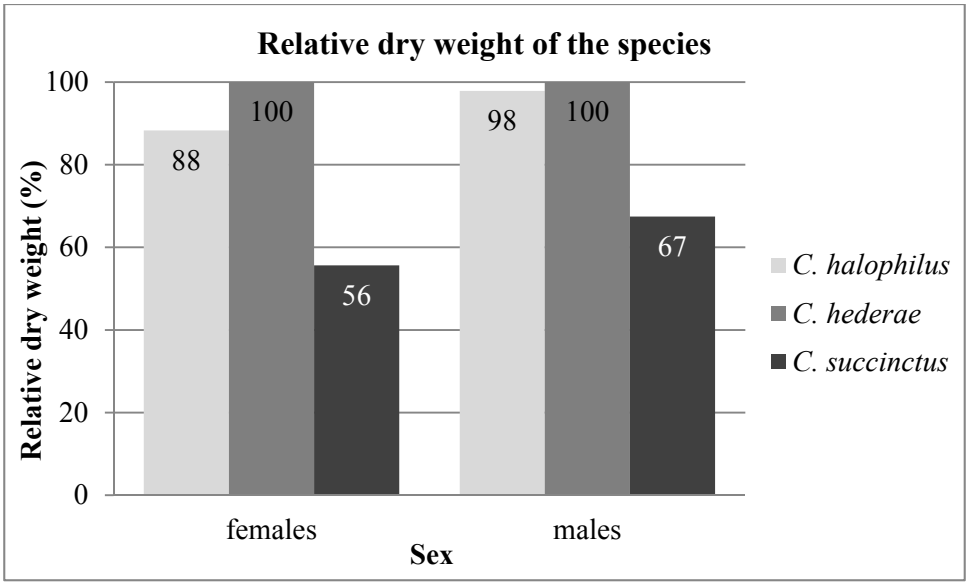

Figure 52. Ratio between the dry weights of the three bee species. For the heaviest species *C. hederae* the weight is taken as 100% to compare the relative weight of the three species.

4.3. Sex of eggs or larvae

81 eggs or small larvae of *C. halophilus*, 51 eggs or small larvae of *C. hederae* and 32 large larvae of *C. succinctus* were analyzed.

Examples of resulting diagrams for a haploid (male) and a diploid (female) sample are shown in Figures 53 and 54.

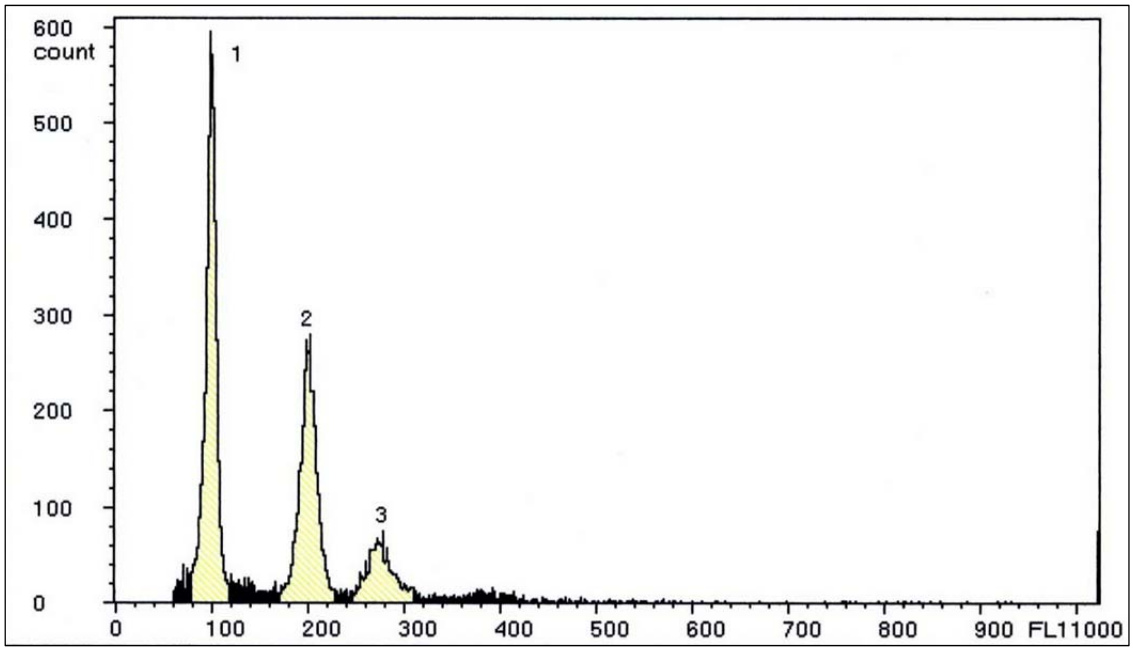

Figure 53. Diagram resulting of flow cytometry showing the peaks of male eggs or larvae. Each peak shows cells with a different type of DNA (haploid, diploid). The x axis shows the detected signal (intensity of coloration). The y axis shows the counted cells. The first peak (100) shows the 'normal' cells with haploid DNA. The second peak (200) shows doubled color intensity. It results from cells which are in division (diploid) and muscle cells which are always diploid.

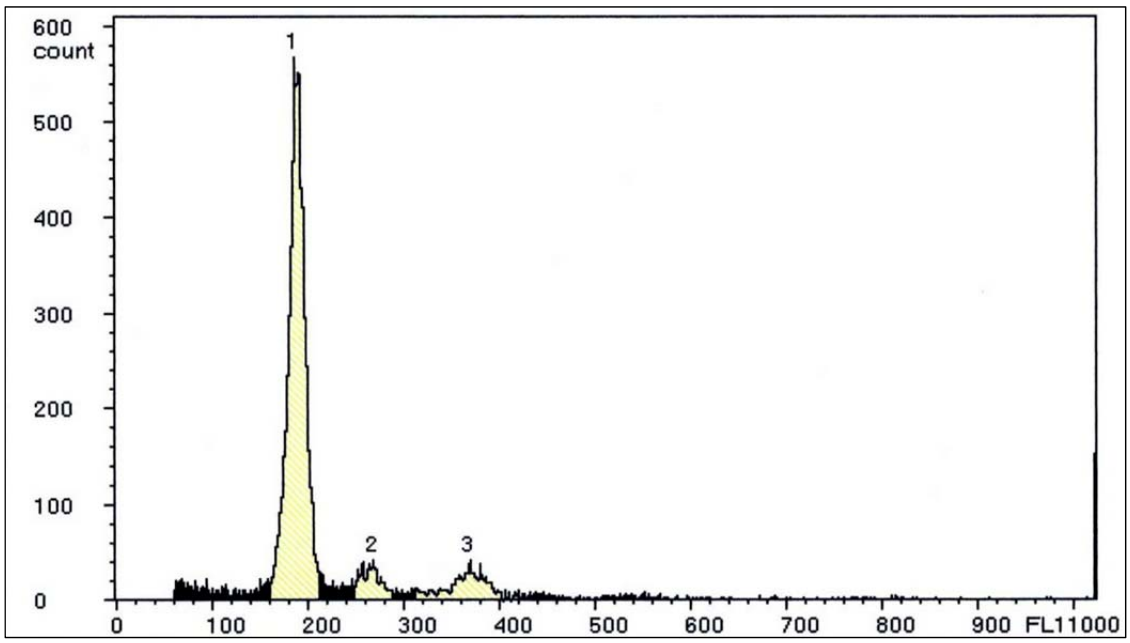

Figure 54. Diagram resulting of flow cytometry showing the peaks of female eggs or larvae. Each peak shows cells with a different type of DNA (haploid, diploid). The x axis shows the detected signal (intensity of coloration). The y axis shows the counted cells. The first peak (200) indicates the diploid DNA of 'normal' cells. The third peak (nearly 400) shows the doubled DNA of diploid cells which are in division.

The results of the sex determination are presented in Table 6. 43 out of 81 eggs and larvae of *C. halophilus* were determined. The results show 11 diploid (female) and 32 haploid (male) individuals. For *C. hederae* 37 out of 51 eggs or larvae (2 diploids/females and 35 haploids/males) were determined. 31 out of the 32 analyzed larvae of *C. succinctus* were determined. 7 of them are diploid (females), 24 are haploid (males).

Table 6. Number of female and male eggs and larvae which could be detected by the flow cytometer.

Species	Sex	Number
Colletes halophilus	female	11
	male	32
Colletes hederae	female	2
	male	35
Colletes succinctus	female	7
	male	24

The flow cytometry of 32% of the samples did not result in evaluable peaks. Probably these eggs or larvae were already dead before the analysis. That would lead to destruction of their cells, which can not be detected by the flow cytometer.

A pollen loaf or brood cell which contained a female egg or larva will be called “female” pollen loaf or brood cell in the following. A pollen loaf or brood cell which contained a male egg or larva will be called “male” pollen loaf or brood cell.

4.4. Weight of pollen loaves

While the pollen loaves of *C. halophilus* and *C. hederae* contained eggs or small larvae, the brood cells of *C. succinctus* contained already large larvae. They were weighed freshly with the larvae inside. Therefore only the dry weight of *C. halophilus* and *C. hederae* can be compared. For females of *C. hederae* only one fresh weight could be measured. The result is included in the diagrams but was not statistically analyzed.

To compare all three species, the fresh weight is used. Therefore the pollen loaves (without egg or larva) of *C. halophilus* and *C. hederae* will be compared to the brood cells (pollen loaf + larva) of *C. succinctus*. Only pollen loaves, of which the sex of the contained egg or larva could be determined, can be considered in this analysis.

4.4.1. Fresh weight

The fresh weight of 38 pollen loaves of *C. halophilus*, 34 pollen loaves of *C. hederae* and 31 brood cells of *C. succinctus* was analyzed (*Tab. 7*, *Fig. 56*, *Appendix 2*).

Table 7. Fresh weight of the pollen loaves of *C. halophilus* and *C. hederae* and of the brood cells of *C. succinctus.* Column 4 to 7: all values are given in mg.

Species	Sex	Number	Mean	SD	Lowest weight	Highest weight
Colletes halophilus	female	n=11	256.71	59.39	121.90	313.80
	male	n=27	170.17	34.18	110.90	275.10
Colletes hederae	female	n=1	261.5	0	-	-
	male	n=33	192.14	29.65	129.30	244.60
Colletes succinctus	female	n=7	104.61	38.69	50.50	158.60
	male	n=24	91.38	25.49	50.20	167.00

Colletes halophilus

The size of the brood cells of *C. halophilus* vary remarkably (observed during collection in 2012, see *Fig. 55*). There were large and heavy brood cells with much pollen provision and smaller, lighter brood cells which less provision.

Figure 55. Brood cells of *Colletes halophilus.* Both are cut open and the eggs are already removed. Left: 131.8mg, male. Right: 316.5mg, sex could not be determined. Collected in 2012 in the Netherlands, Saeftinghe.

The fresh weight of female pollen loaves of *C. halophilus* ranges from 122mg to 314mg (*Tab. 7*). A mean weight of 257mg with a standard deviation of 59mg (*Fig. 56*) was gained across the 11 females. Male pollen loaves have a fresh weight of 111mg to 275mg. The mean weight of the 27 measured males is 170mg; the standard deviation is 34mg.

Colletes hederae

The measured female pollen loaf of *C. hederae* has a weight of 261.5mg (*Tab. 7*). The majority of results of the 33 male pollen loaves are in the range between 129mg and 245mg. On average the considered male pollen loaves weigh 192mg; the standard deviation is 30mg (*Fig.56*).

Colletes succinctus

The results of the 7 analyzed female brood cells of *C. succinctus* are between 50.5mg and 159mg (*Tab. 7*). The mean value is 105mg and the standard deviation is 39 (*Fig. 56*). For the male brood cells the fresh weight ranges from 50mg to 167mg. The mean weight is 91mg (n=24) with a standard deviation of 25mg.

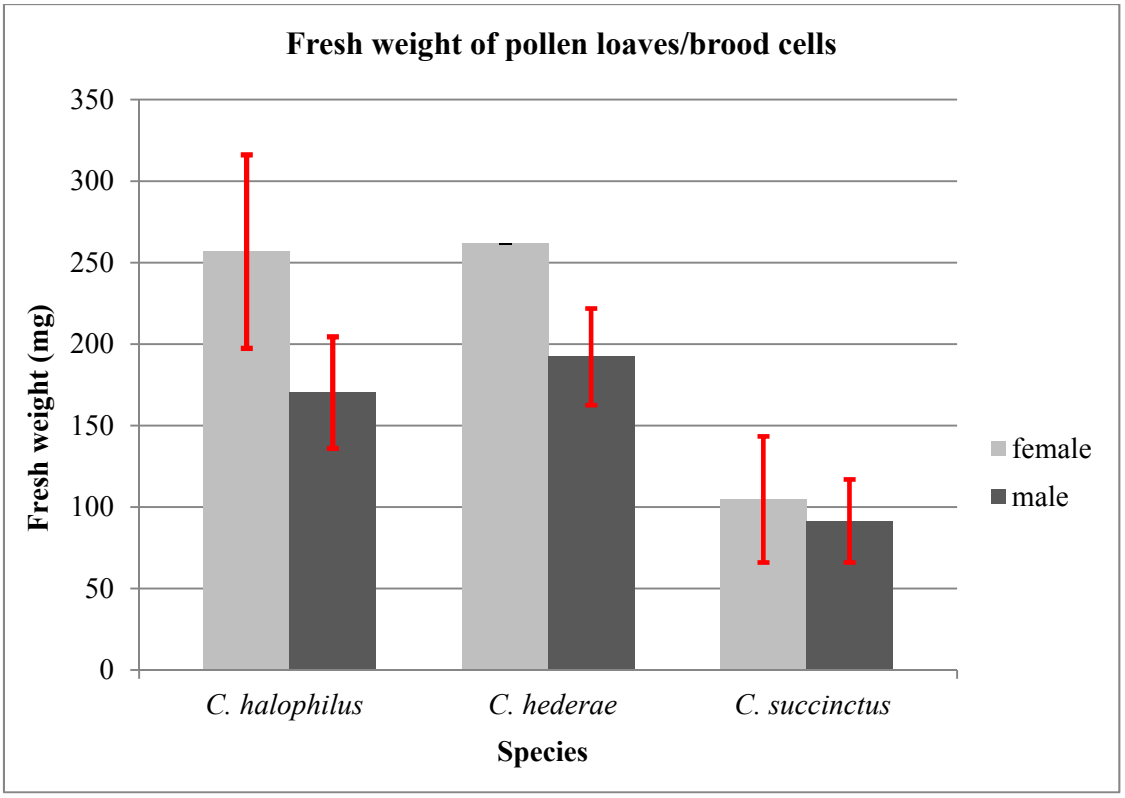

Figure 56. Fresh weight of the pollen loaves/brood cells. The mean value of the fresh weight is illustrated by bars. The standard deviation is marked by red indicators.

Intraspecific variability

Within all species the female pollen loaves/brood cells are heavier than the male pollen loaves/brood cells (*Fig. 57*). The male pollen loaves of *C. halophilus* are on average 34% lighter than the females. The males of *C. hederae* are on average 27% lighter than the female

pollen loaves. The female brood cells of *C. succinctus* are on average 13% heavier than the males.

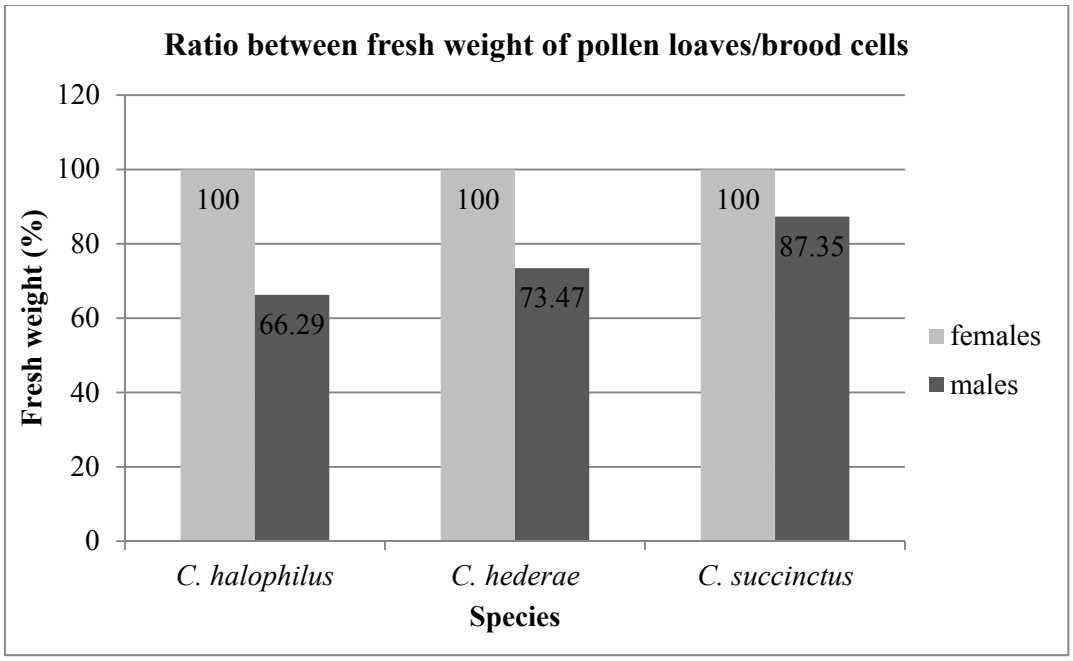

Figure 57. Ratio between the fresh weight of male and female pollen loaves/brood cells. For each species the mean fresh weight of the females is taken as 100% to compare the relative weight of both sexes within a species.

The values for *C. halophilus* and *C. succinctus* are normally distributed. For the logarithm of the values homogeneity of variances could be proved. The difference between the sexes within *C. halophilus* is significant (see *Fig. 58*). But there is no significant difference between the sexes of *C. succinctus*.

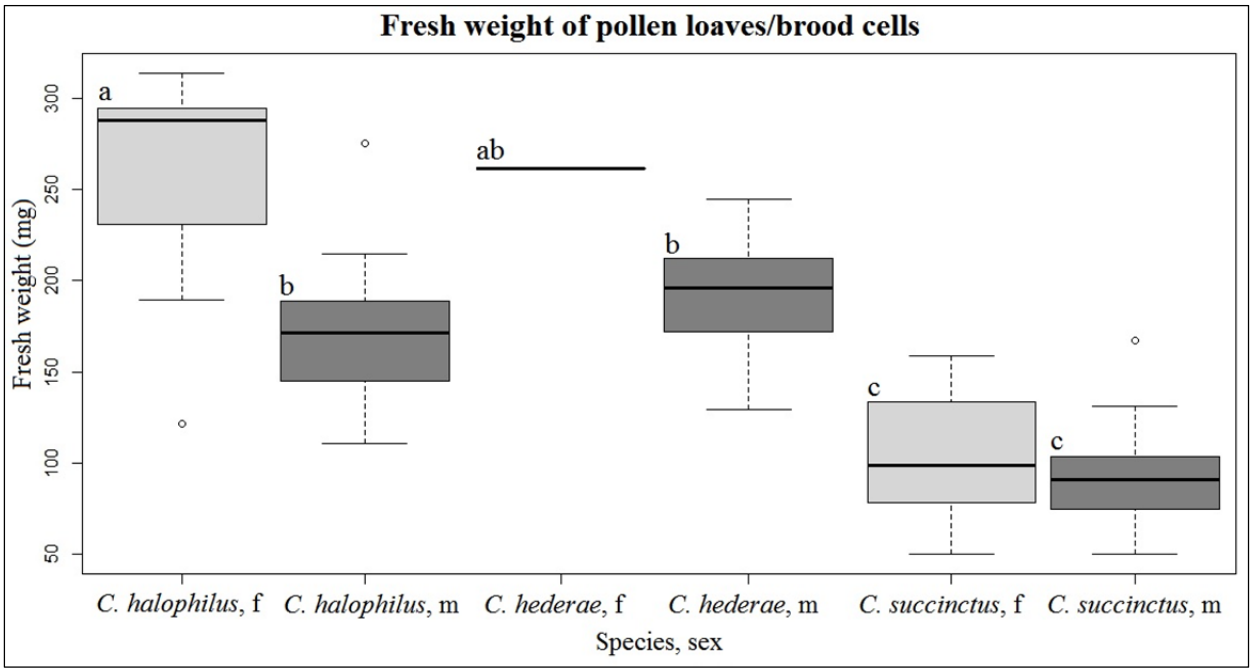

Figure 58. Boxplot diagram showing the fresh weight of pollen loaves/brood cells. It shows the differences between male and female pollen loaves of all three species. Light grey: females. Dark grey: males. The pollen loaves/brood cells which differ significantly from each other are labeled with different small letters.

Interspecific variability

The fresh weight of the pollen loaves of *C. halophilus* and *C. hederae* are compared to the fresh weight of the total brood cells of *C. succinctus*. That is possible due to the fact that the brood cells of *C. succinctus* are very much lighter than the pollen loaves of *C. halophilus* and *C. hederae*. During larval growth only air of respiration can permeate the cell lining of the brood cell. Due to this fact, the entire brood cell can not have been much heavier before the larva started to consume the pollen loaf.

C. hederae has the heaviest female pollen loaves (*Fig. 59*). The female pollen loaves of *C. halophilus* are 2% lighter. The females of *C. succinctus* are on average 60% lighter than *C. hederae*. The heaviest male pollen loaves are equally those of *C. hederae*. They are on average 11% heavier than the males of *C. halophilus* and 52% heavier than the male brood cells of *C. succinctus*.

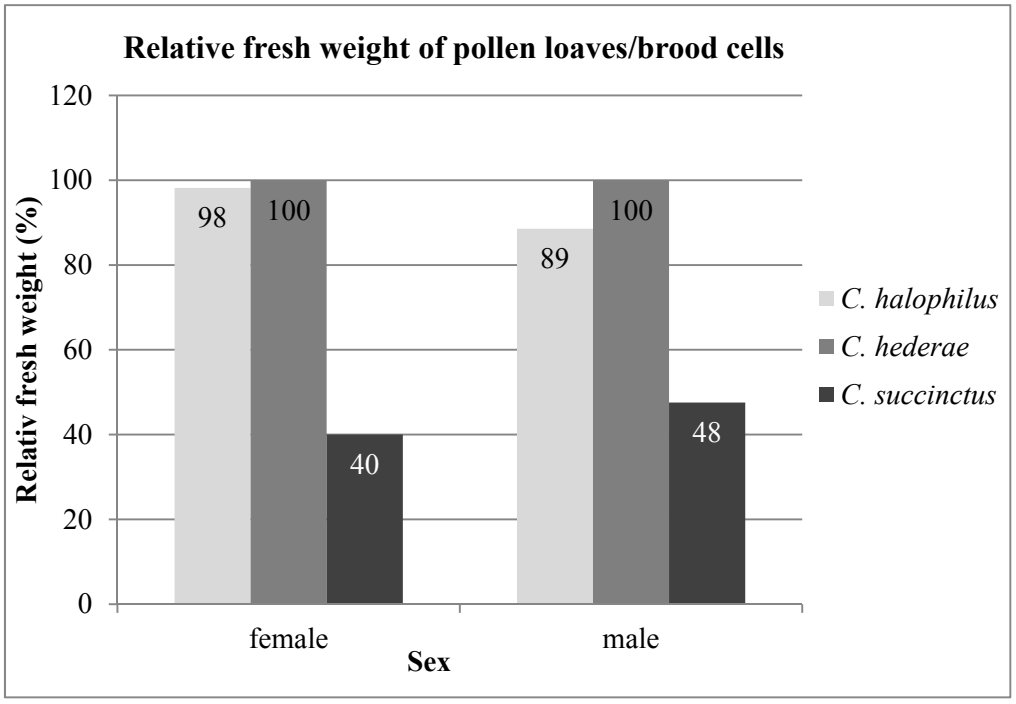

Figure 59. Relative fresh weight of the pollen loaves/brood cells. The diagram shows the ratio of female and male pollen loaves/brood cells between all species. For both sexes the *C. hederae* is taken as 100% to illustrate the difference between the species.

For *C. hederae* only one female was measured. It seems that it is not significantly different from *C. halophilus* and the male of *C. hederae*. The logarithm of the values for the females of *C. halophilus* and *C. succinctus* are normally distributed. Homogeneity of variances is proved for the logarithm. The differences between *C. halophilus* and *C. succinctus* and between *C. hederae* and *C. succinctus* are significant (see *Fig. 58*). The values for the males of the three species are normally distributed and homogeneity of variances was proved. The difference between males of *C. halophilus* and *C. hederae* is not significant (*Fig. 58*).

4.4.2. Dry weight

As the larvae in the brood cells of *C. succinctus* were already very large and had already started to consume the pollen loaf, the dry weight of the entire pollen loaf could not be measured. The dry weight was only measured for *C. halophilus* and *C. hederae*.

For some of the samples, the dry weight could not be measured due to the nectar fluidity of the pollen loaves being stuck in the tubes after lyophilization. The dry weight of 32 pollen loaves of *C. halophilus* and 29 pollen loaves of *C. hederae* was measured (*Tab. 8*, *Fig. 60*). Only for one of the two determined female pollen loaves of *C. hederae* the dry weight could be measured.

Table 8. Dry weight of the pollen loaves of *C. halophilus* and *C. hederae.* Column 4 to 7: all values are given in mg.

Species	Sex	Number	Mean	SD	Lowest weight	Highest weight
Colletes halophilus	female	n=11	213.6	49.544	101.10	262.90
	male	n=21	137.5	29.008	91.60	210.40
Colletes hederae	female	n=1	166.6	-	-	-
	male	n=28	130.5	18.455	91.20	165.90

Colletes halophilus

The dry weight of the 11 female pollen loaves of *Colletes halophilus* ranges from 101.1mg to 262.9mg (*Tab. 8*). The mean is at 213.6mg with a standard deviation of 49.5mg (*Fig. 60*). The results for male pollen loaves are between 91.6mg to 210.4mg. The mean value of the 21 measured males is 137.5mg; the standard deviation is 29mg.

Colletes hederae

The dry weight of the female pollen loaf of *C. hederae* is 166.6mg (*Tab. 8*). The majority of values for the 28 males are in the range between 91.2mg and 165.9mg. On average the considered male pollen loaves weigh 130.5mg (*Fig. 60*); the standard deviation is 18.5mg.

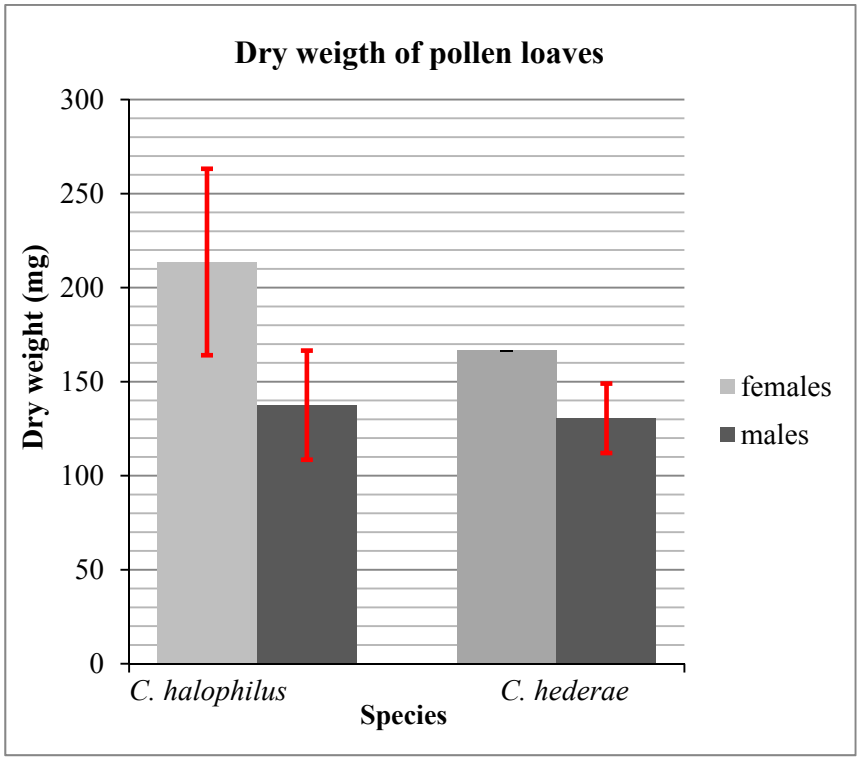

Figure 60. Dry weight of the pollen loaves. Male and female pollen loaves of *C. halophilus* and *C. hederae* are shown. The mean value of the dry weight is illustrated by bars. The standard deviation is marked by red indicators.

Intraspecific variability

The values are normal distribution when the parameter "sex" is excluded from the analysis. Homogeneity of variances is not present.

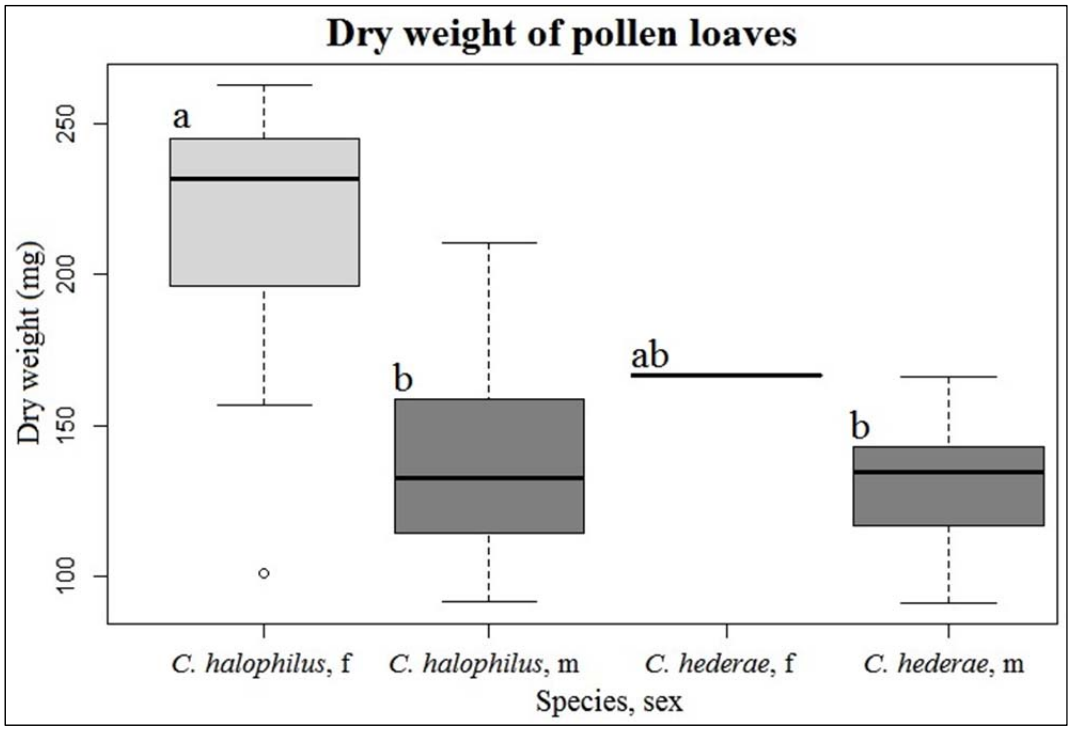

Figure 61. Boxplot diagram showing the differences of the logarithm of the dry weight between male and female pollen loaves of *C. halophilus* and *C. hederae*.

Within *C. halophilus* the female pollen loaves are heavier than the male pollen loaves. On average the dry weight of the male pollen loaves is 36% less than the dry weight of the female pollen loaves (*Fig. 62*). The values are normally distributed and the homogeneity of variances is proved. The difference in the dry weight between male and female pollen loaves is significant (*Fig. 61*).

The difference between female and male pollen loaves of C. hederae seems not to be significant. Statistical analyses were not carried out.

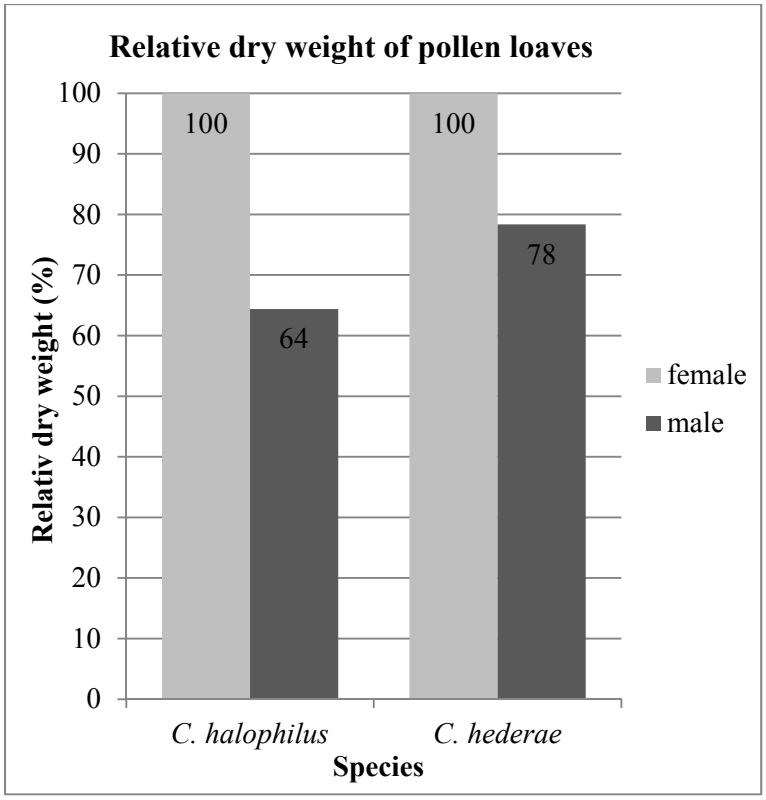

Figure 62. Ratio between the dry weight of male and female pollen loaves. The mean of the dry weight of the female pollen loaves is taken as 100% to compare the relative weight of both sexes.

Interspecific variability

The female pollen loaves of *C. halophilus* are 22% heavier than females of *C. hederae* (*Fig. 63*). The difference was not statistically analyzed. The male pollen loaves of *C. halophilus* are 5% heavier than the male pollen loaves of *C. hederae* (*Fig. 63*). The values of the dry weight of the pollen loaves are normally distributed and homogeneity of variances is proved, but the small difference between the males of the two species is not significant (*Fig. 61*).

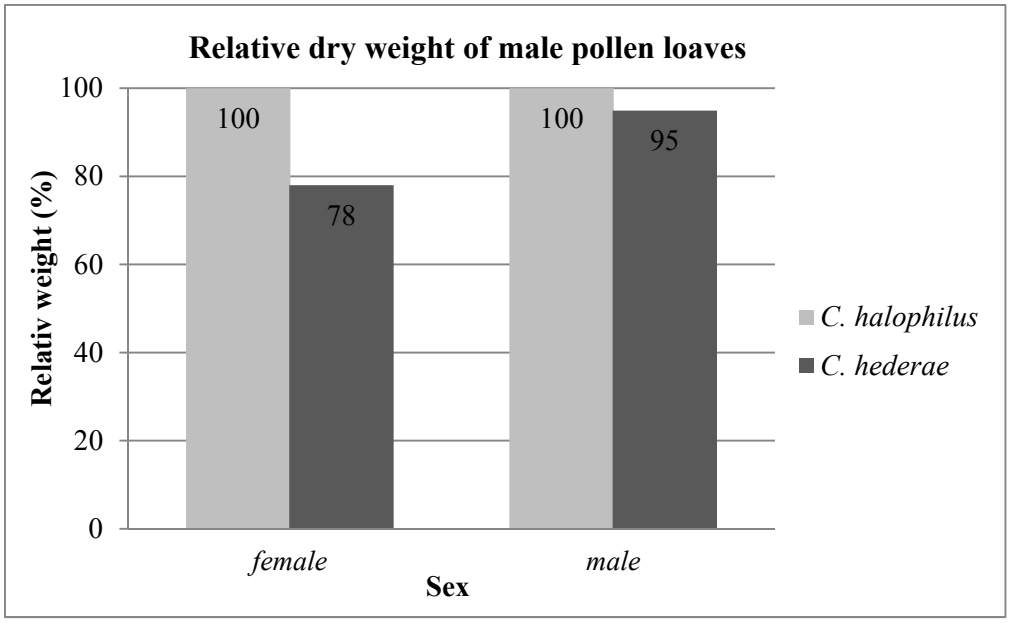

Figure 63. Ratio between the dry weights of male pollen loaves of *C. halophilus* and *C. hederae*. For *C. halophilus* the mean dry weight is taken as 100% to compare the difference to *C. hederae*.

4.5. Quantity of polypeptides in pollen loaves

The polypeptide quantity of 17 pollen loaves was measured. For *C. halophilus* and *C. succinctus* 3 male and 3 female pollen loaves were analyzed (*Tab. 9*, *Fig. 64*). For *C. hederae* 3 male and 2 female pollen loaves were considered. The polypeptide concentration (µg/mg) was calculated for the dry weight of the pollen loaves.

Table 9. Polypeptide concentration of the pollen loaves. The table shows the polypeptides amount (µg) contained in 1mg dry weight of the analyzed pollen loaf. Column 4: mean value of the three analyzed pollen loaves per sex. Column 5: Standard deviation. Column 6: Polypeptide concentration in %. For *C. hederae* only two female pollen loaves were available.

Species	Sex	Number	Mean (µg/mg)	SD	Polypeptide concentration (%)
Colletes halophilus	female	n=3	73.05	5.78	7.30
	male	n=3	70.17	4.46	7.02
Colletes hederae	female	n=2	73.45	6.78	7.34
	male	n=3	79.55	4.50	7.95
Colletes succinctus	female	n=3	117.90	14.46	11.79
	male	n=3	114.05	20.39	11.41

Colletes halophilus

Female pollen loaves of *C. halophilus* contain on average 73µg polypeptides per mg dry weight (*Fig. 64*). The standard deviation of the values is 5.78µg/mg. The mean for male pollen loaves is about 70µg per mg dry weight. A standard deviation of 4.46µg/mg was measured.

Colletes hederae

The female pollen loaves of *C. hederae* contain on average 73.45µg polypeptides per mg dry weight (*Fig. 64*). The standard deviation is 6.78µg/mg. The mean value for male pollen loaves is 79.55µg/mg; with a standard deviation of 4.5µg/mg.

Colletes succinctus

The pollen loaves of *C. succinctus* contain on average 117.9µg polypeptides per mg dry pollen loaf (females) and 114µg/mg (males) respectively (*Fig. 64*). The standard deviations are 14.46µg/mg for the females and 20.39µg/mg for the males.

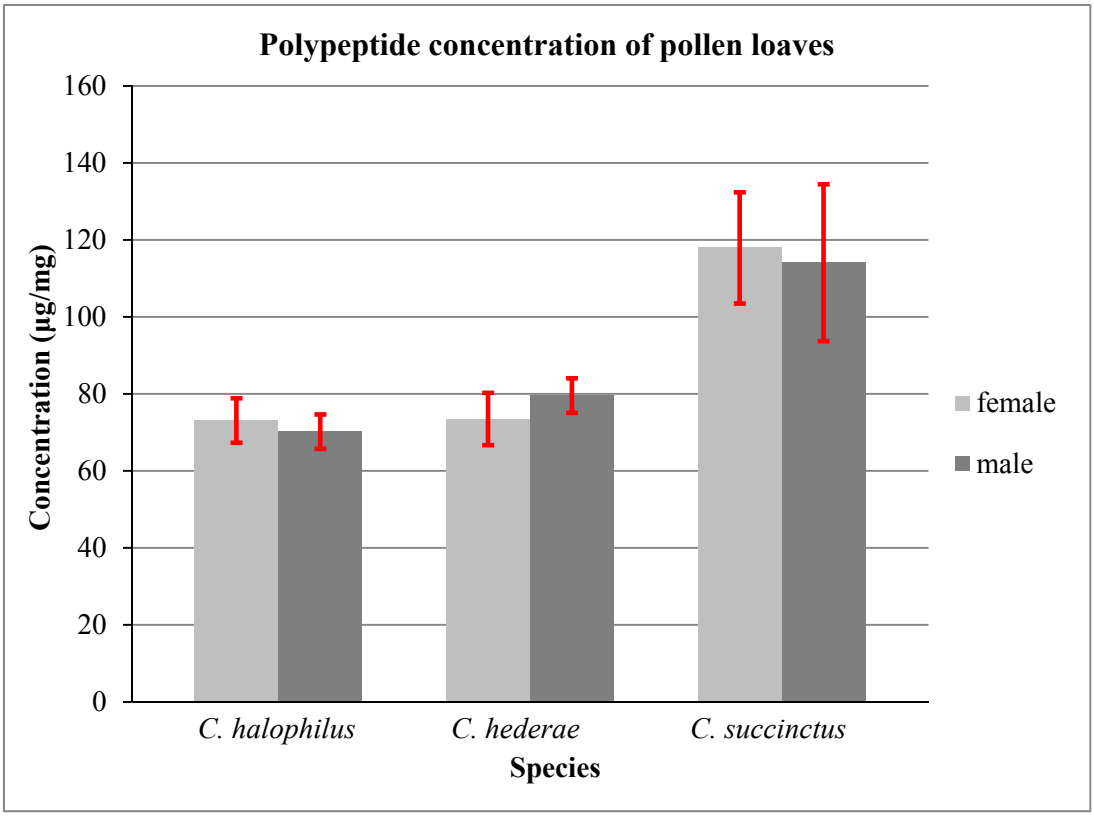

Figure 64. Concentration of polypeptides in pollen loaves. The bars show the mean values of the polypeptide concentration per mg dry weight of the pollen loaves. The standard deviation is marked by red indicators.

Intraspecific variability

The resulting values for the polypeptide concentration per mg dry weight are normally distributed. Homogeneity of variances could be proved for the logarithm of the values. Further statistical analyses were all carried out with the logarithm of the measuring results.

The difference between the polypeptide concentrations of male and female pollen loaves is not significant in all species (*Fig. 65*).

Interspecific variability

There is no significant difference between male or female pollen loaves of *C. halophilus* and *C. hederae* (*Fig. 65*). Only the polypeptide concentration of male and female pollen loaves of *C. succinctus* differ significantly from the other male and female pollen loaves.

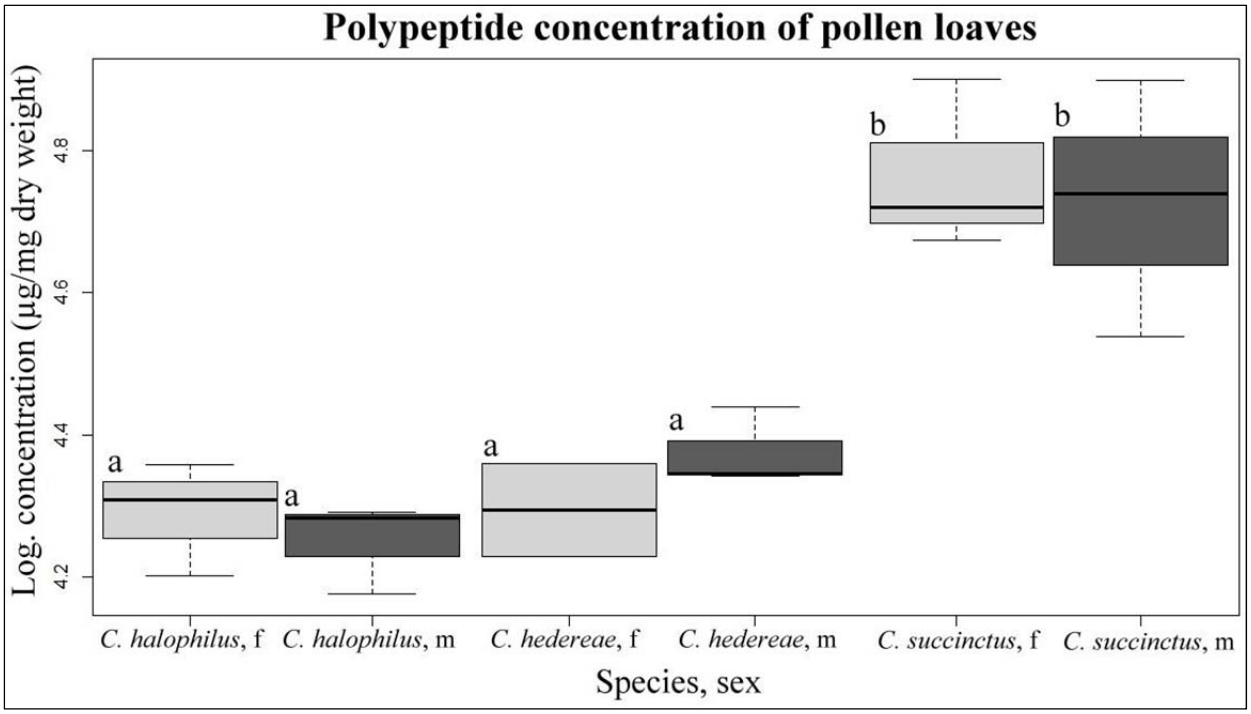

Figure 65. Boxplot diagram showing differences in the polypeptide concentration per mg dry weight of the pollen loaves. Light grey: female pollen loaves. Dark grey: male pollen loaves. The results which differ significantly from each other are labeled with different small letters.

4.6. Comparisons

4.6.1. Comparison of size and weight of adult bees

A comparison of the dry weight and the wing size of the three colletid bee species is illustrated in Figure 66. The results show that the largest individuals (females of *C. hederae*) are equally the heaviest individuals, and that the smallest individuals (males of *C. succinctus*) are equally the lightest individuals. There is only one exception: the males of *C. hederae* are larger than the females of *C. succinctus* but they are much lighter (see *Fig. 66*).

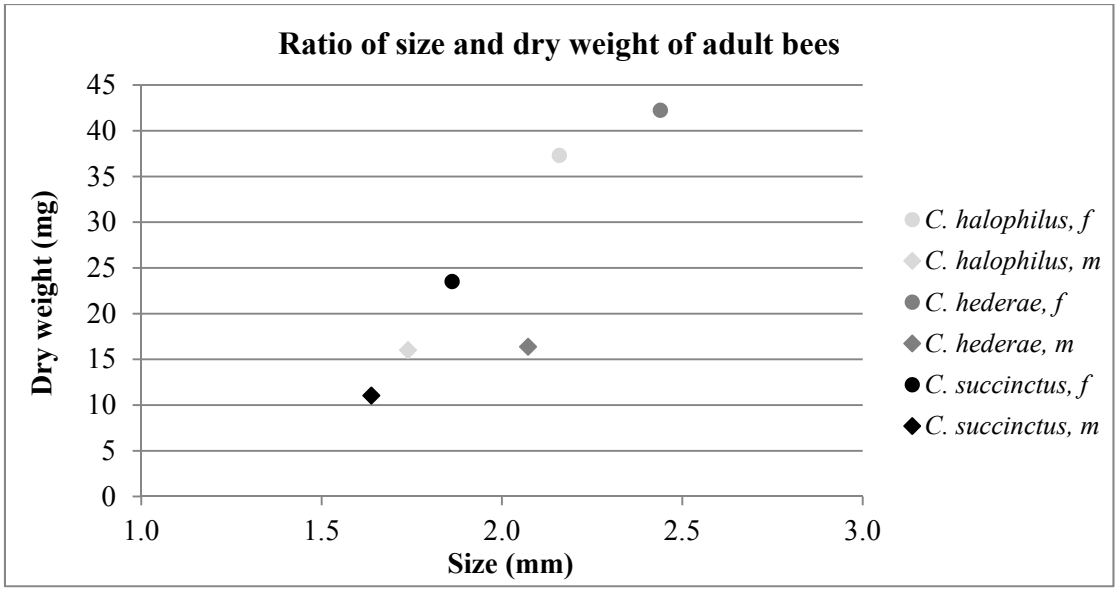

Figure 66. Comparison of size and dry weight of adult bees. The dots show the mean value of size and weight for the females of each species. The squares indicate the males; the dots indicate the females. “Size”: mean distance between landmarks 1 and 3 on the right anterior wing of the bees.

4.6.2. Comparison: fresh and dry weight of pollen loaves

C. succinctus is not included in this comparison because the larvae were already too large and the dry weight was not measured. The relation of the fresh and dry weight of the pollen loaves of *C. halophilus* and *C. hederae* are considered in this comparison (*Tab. 10*, *Fig. 67*). For the female pollen loaves of *C. hederae* there was only one value for the weight. Male and female pollen loaves of *C. hederae* have a higher fresh weight, but a lower dry weight than their counterparts of *C. halophilus* (*Tab. 10*, *Fig. 67*).

Table 10. Weight of pollen loaves of *Colletes halophilus* and *Colletes hederae*. For *C. hederae* only one female pollen loaf was analyzed.

Species	Sex	Number	Mean fresh weight (mg)	Mean dry weight (mg)	Mean dry weight (%)	SD (%)
Colletes halophilus	female	n=11	256.7	213.6	83.2	2.6
	male	n=21	168.9	137.5	81.5	2.5
Colletes hederae	female	n=1	261.5	166.6	63.7	-
	male	n=28	190.0	130.5	69.1	4.3

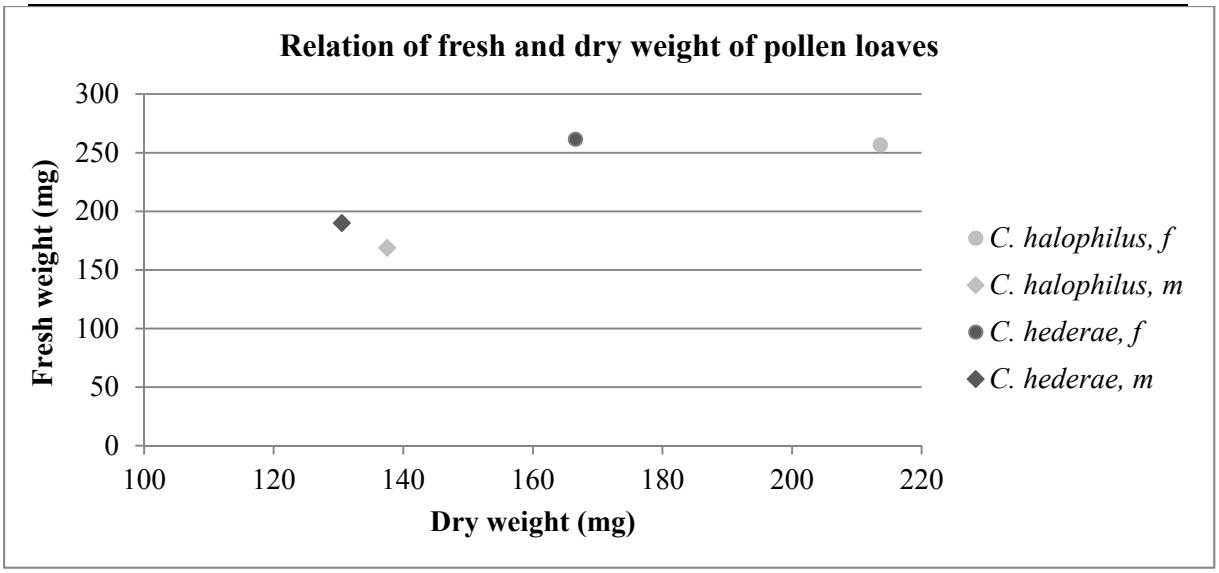

Figure 67. Relation between fresh and dry weight of pollen loaves. Female pollen loaves are indicated by dots and males are indicated by squares.

The values for the relative dry weight of the considered species and sexes are normally distributed (when female *C. hederae* is excluded). Homogeneity of variances was proved (when female *C. hederae* is excluded). No statistical analyzes were made for the single *C. hederae* female pollen loaves, because one result is not representative for the whole species.

Intraspecific variability

The relative dry weight of female pollen loaves of *C. halophilus* is on average 83% (*Fig. 68*). The standard deviation is 2.6%. The mean of the relative dry weight of the male pollen loaves is only 81.5% with a standard deviation of 2.5%. But the difference between males and females is not significant (see *Fig. 69*).

The female of C. hederae has a relative dry weight of 64% (Fig. 68). The mean dry weight for the males of C. hederae is 69%. The standard deviation is 4.3%. No statistical analyses were made for the female pollen loaf.

Interspecific variability

Only the male pollen loaves are considered for this analysis. Male pollen loaves of *C. hederae* have a relative dry weight of 69% with a standard deviation of 4.3%. They have a lower relative dry weight than *C. halophilus* (81.5%, *Fig. 68*). The difference between the relative dry weight of males of *C. halophilus* and *C. hederae* is significant (see *Fig. 70*).

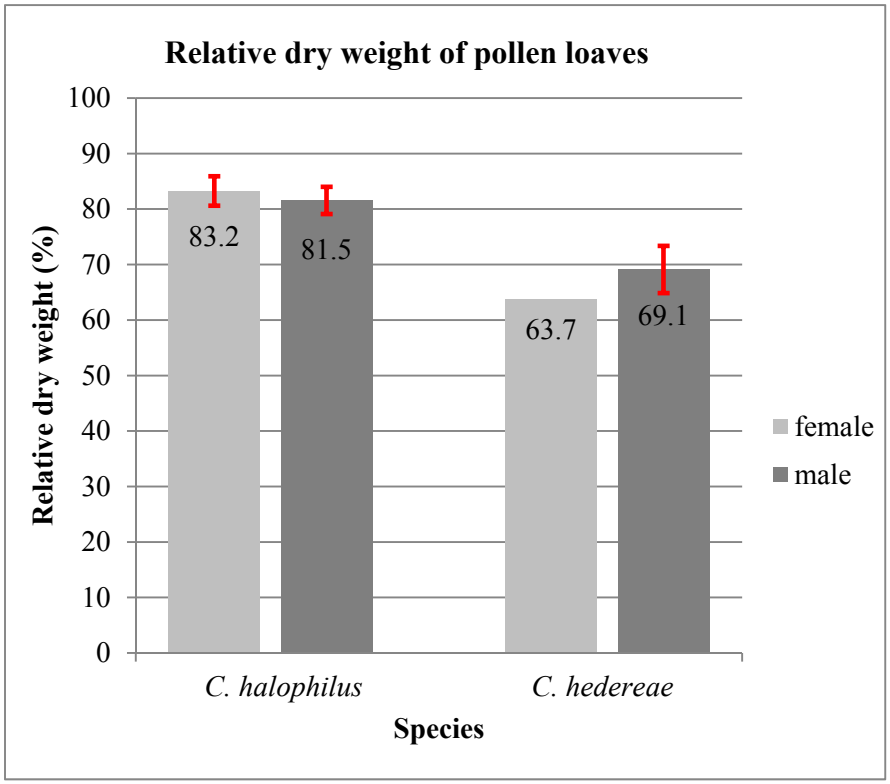

Figure 68. Relative dry weight of pollen loaves. The diagram shows the mean value of the relative dry weight of all analyzed pollen loaves of *C. halophilus* and *C. hederae*. The shown value for *C. hederae* female pollen loaves results from one single measure. For each species and sex the fresh weight was taken as 100%.

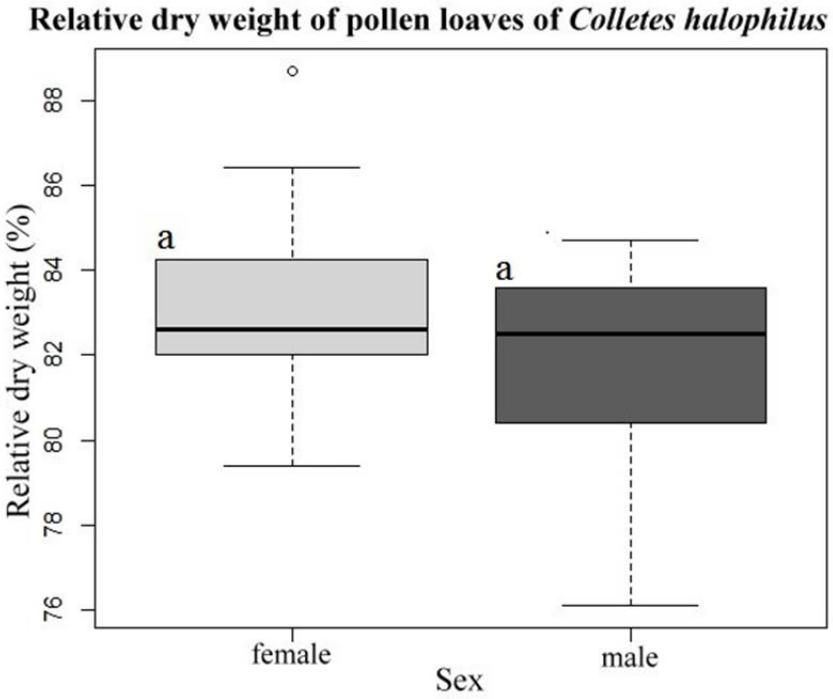

Figure 69. Boxplot diagram showing the relative dry weight of pollen loaves. Comparison between female and male pollen loaves of *C. halophilus*.

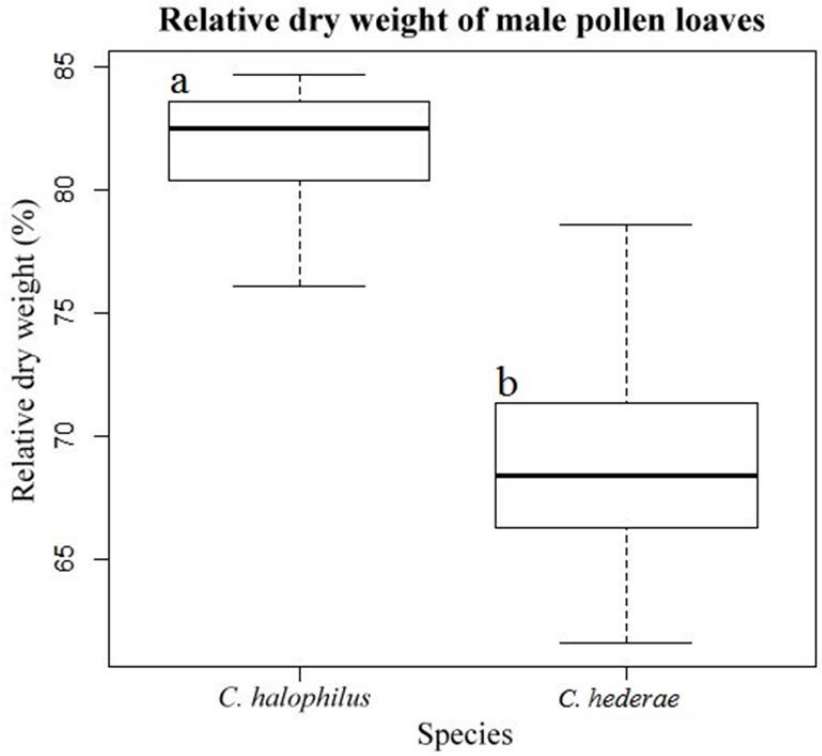

Figure 70. Boxplot diagram showing the relative dry weight of pollen loaves. Comparison of male pollen loaves between *C. halophilus* and *C. hederae*.

4.6.3. Comparison: female adults and the weight of their pollen loaves

Due to the fact that weight can change after nectar uptake the size of the female bees is used to compare females and the pollen loaves they provide for their offspring (*Fig. 71*). For the pollen loaves/brood cells the fresh weight is considered. For *C. hederae* there was only one female pollen loaf.

The females of *C. hederae* are significantly heavier than the females of *C. halophilus*. However, the size of their pollen loaves is similar (no significant difference). The smallest females (*C. succinctus*) build the lightest brood cells (for male and female eggs).

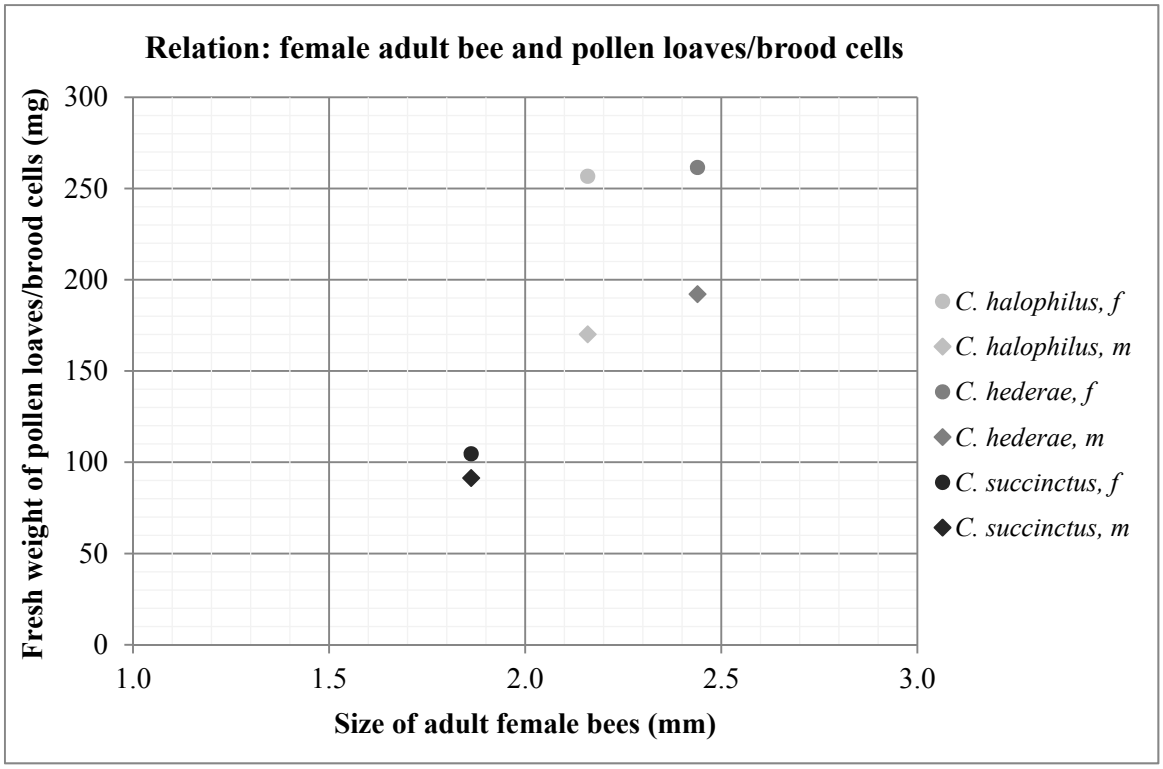

Figure 71. Relation between female size and weight of pollen loaves/brood cells. The x axis represents the mean distance between landmarks 1 and 3 of the wings of all female adults of a species. The y axis shows the mean fresh weight of all male or female pollen loaves/brood cells of a species. The dots show the fresh weight for female pollen loaves/brood cells; the squares show the male pollen loaves/brood cells.

4.6.4. Comparison: weight of pollen loaves and size of adult bees

The largest adult bees develop from the heaviest pollen loaves (*Fig. 72*). The female pollen loaves of *C. hederae* are on average heavier (significance tested for males) than the pollen loaves of *C. halophilus* and the female bees of *C. halophilus* are on average smaller (significantly) than the female bees of *C. hederae*. *C. succinctus* has the lightest (significantly) brood cells, but their female adults are larger (significant difference) than the male adults of *C. halophilus* (significant difference). The male brood cells as well as the male adult bees of *C. succinctus* are significantly smaller than all other pollen loaves/brood cells and adult bees.

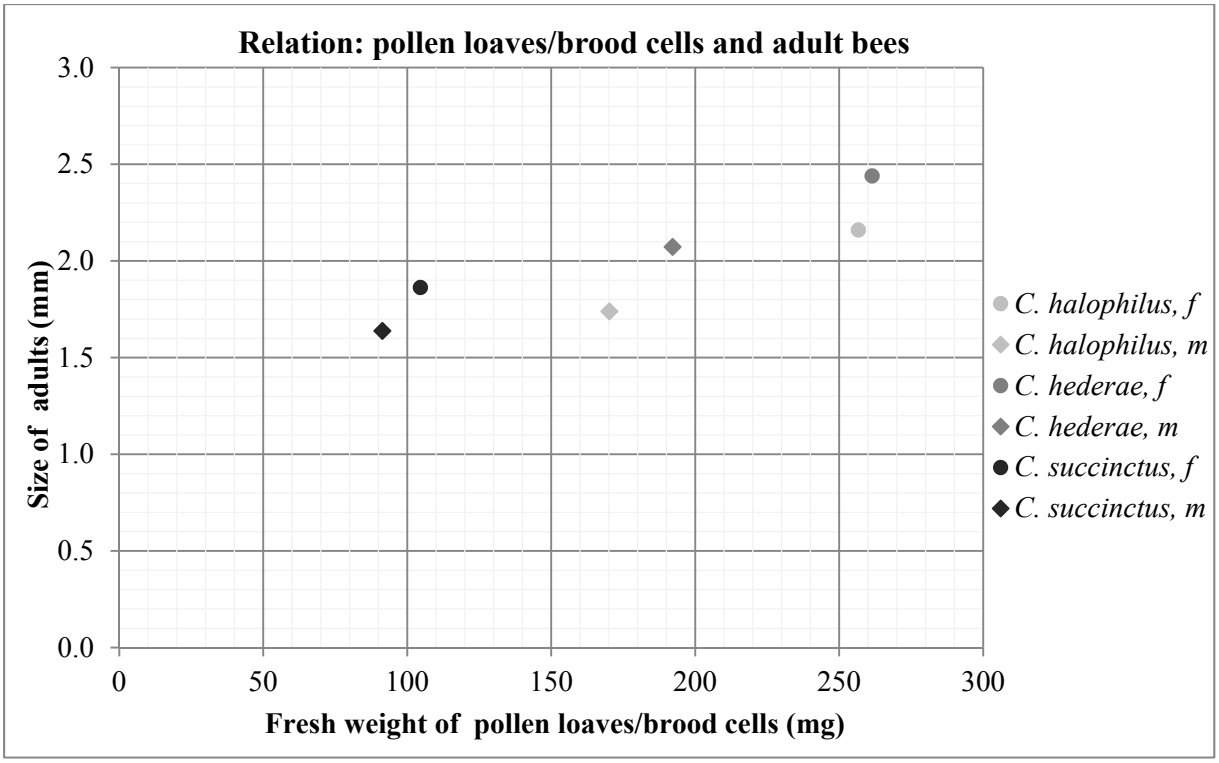

Figure 72. Relation between pollen loaves and adult bees of the same species. The x axis represents the mean fresh weight of all male or female pollen loaves/brood cells of a species. The y axis shows the mean distance between landmarks 1 and 3 on the wings of all male or female adults of a species. Female pollen loaves/brood cells are compared to female adults (dots) and male pollen loaves/brood cells are compared to male adults (squares). For *C. hederae* there were only two female pollen loaves.

4.6.5. Comparison: total polypeptide quantity in pollen loaves and size of adult bees

The relation between total polypeptide content of the pollen loaves and adult size is presented in Table 11 and Figure 73. For *C. hederae* the total polypeptide concentration was calculated for only one female pollen loaf. For *C. succinctus* the dry weight of pollen loaves could not be measured, therefore the total polypeptide quantity could not be calculated.

Table 11. Total polypeptide content of pollen loaves and wing size of adult bees.

Species	Sex	Total polypeptide content (mg/loaf)	Adult wing size (mm)
Colletes halophilus	female	17.28 (n=3)	2.16 (n=45)
	male	10.99 (n=3)	1.74 (n=42)
Colletes hederae	female	13.04 (n=1)	2.44 (n=51)
	male	8.85 (n=3)	2.07 (n=67)

Female pollen loaves of *C. halophilus* contain on average 17.28mg polypeptides (*Tab. 11*). They contain 36% more polypeptides than their male counterparts (10.99mg). The female pollen loaf of *C. hederae* contains 13.04mg polypeptide, the male pollen loaves contain on average 8.85mg polypeptides. The female pollen loaf contains 32% more polypeptides.

There is a no correlation between polypeptide content of pollen loaves and adult size (*Fig. 73*). The pollen loaves of *C. halophilus* contain the more total polypeptides, but their adult size is smaller than in *C. hederae*.

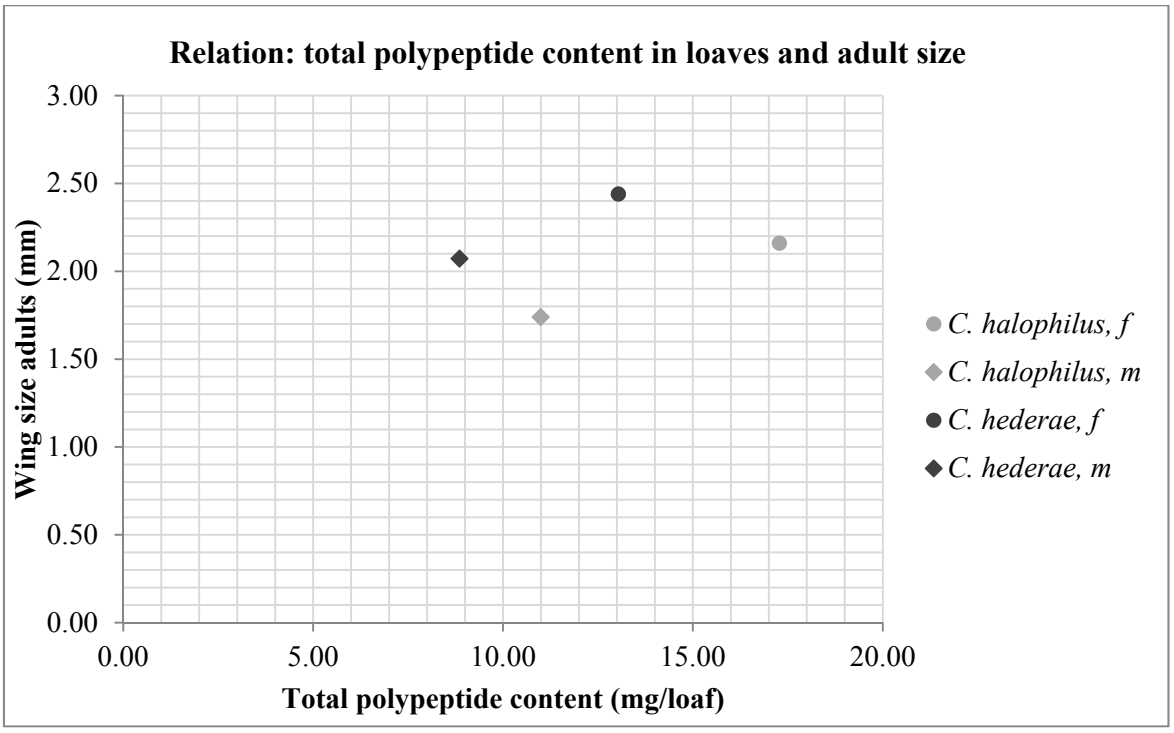

Figure 73. Relation between total polypeptide content in pollen loaves and adult size. The x axis represents the mean total polypeptide content of the male and female pollen loaves. The y axis shows the mean wing size of all male or female adults of a species. Female pollen loaves are compared to female adults (dots) and male pollen loaves/brood cells are compared to male adults (squares). For *C. hederae* there was only one female pollen loaf.

5. Discussion

5.1. Adult bees – sexual dimorphism

The herein analyzed adult bees of *C. halophilus*, *C. hederae* and *C. succinctus* show an explicit sexual dimorphism in size and weight. The females of *C. halophilus* are on average 19.5% larger and 57% heavier than males. Females of *C. hederae* are on average 15% larger and 61% heavier than the male bees. Females of *C. succinctus* are on average 12% larger and 53% heavier than males. Therefore sexual dimorphism in adults is less in *C. succinctus*. Sexual size dimorphism within these species was already reported by VERHOEFF (1943) and SCHMIDT & WESTRICH (1993). However, the results show that the relation between size and weight does not correlate stringently. Specimens which foraged on flowers are heavier, due to their crop full of nectar. But their size does not change.

Sexual size dimorphism (i.e. larger females than males) is common in bees, as well as in other hymenopterans. Within ants there can be as extreme sexual size differences that males and females were classified into different species in the past. The sexual dimorphism in wasps and bees is much smaller: males rarely have a weight less than the half of a female (STUBBLEFIELD & SEGER 1994). The sexual dimorphism results from different functions of male and female individuals (STUBBLEFIELD & SEGER 1994). In general females inherit the construction of nests and the provision of the offspring, what consumes a lot of energy and what is easier to conduct for larger females. Furthermore they have to carry the eggs in their ovaries. Due to the fact that the exoskeleton of insects is not elastic, the number of eggs that they can carry is limited (STEARNS 1977). Therefore a larger body size is beneficial for the reproduction quantity. In contrast males have short lives, serve only as mating partners and do not inherit any parental care (STUBBLEFIELD & SEGER 1994). Therefore their body size can be smaller.

SHREEVES & FIELD (2008) analyzed the sexual dimorphism of 50 wasp taxa (Pompilid wasps, Apoid wasps) and 86 bee taxa (Halictidae, Megachilidae, Apidae). Nearly all females were larger than males. Sexual size dimorphism was larger in wasps than in bees. Female wasps were on average 20% larger than male wasps. Female bees in general were on average 7.4% larger than male bees. Furthermore SHREEVES & FIELD (2008) differentiated between provisioning and non-provisioning (i.e. cuckoo bee) species. Females of provisioning species construct nests (e.g. digging burrows in the soil) or prepare preexisting burrows, and they carry pollen and nectar to their nests to provide their offspring. In contrast females of non-provisioning species deposit their eggs on prey or in the nests of other species. Hence they need no ability to construct nests. Sexual size dimorphism was higher in provisioning than in non-provisioning species. Within non-provisioning species females were on average only 4.8% larger than males. Females of provisioning species were on average 9.1% larger than males. The size difference between male and female bees of provisioning species ranged from 4.8% to 15.5%. The three *Colletes* species belong to provisioning bees. Their sexual size

dimorphism is higher than the average sexual size dimorphism of provisioning bees. In *C. halophilus* it is even higher than the range.

5.2. Female investment in pollen loaf provision

5.2.1. Intraspecific

Pollen loaves of *C. halophilus* show sexual dimorphism. Female pollen loaves are remarkably larger than male pollen loaves, which is consistent with the strong sexual dimorphism of imago. The fresh female pollen loaves are on average 34% heavier than fresh male pollen loaves. The results confirm ROOIJAKKERS & SOMMEIJER (2009), who found that female brood cells are larger in height and diameter, and are approximately 44% heavier than male pollen loaves. The difference between the pollen loaves of *C. halophilus* is less than described by ROOIJAKKERS & SOMMEIJER (2009). In *C. hederae* there seems to be a sexual dimorphism, but that could not been examined. In *C. succinctus* brood cells there is no sexual dimorphism.

The polypeptide concentration is similar between male and female pollen loaves. However the total polypeptide content which is available for the female larvae of *C. halophilus* is higher than for male larvae due to the higher amount of provision in female brood cells. Pollen-nectar mixture is similar between females and males. However that could not been measured for *C. succinctus*, it is supposed to be similar, due to the fact that fresh weight and protein concentration do not differ.

It seems therefore that females do not change their behavior while providing male or female brood cells. However the female investment may differ between provision of female and male brood cells (i.e. weight of pollen loaves). These findings conform to RIBEIRO et al. (1999) who observed differences in frequency of feeding processes (i.e. female investment) between female and male offspring of *Bombus terrestris*.

5.2.2. Interspecific

The polypeptide concentration of the main host plant pollen of *C. halophilus*, *C. hederae* and *C. succinctus* (i.e. *Aster tripolium*, *Hedera helix* and *Calluna vulgaris*) was analyzed by VANDERPLANCK et al. (submitted). All three pollen have a very similar polypeptide concentration of approximately 13.5% (*Tab. 12*). The difference between the pollen is not significant. Differences in total polypeptide quantity of pollen loaves between the species can only be funded in different female investment (i.e. larger or smaller pollen loaves) or different foraging strategy (i.e. different pollen-nectar mixture).

Table 12. Polypeptide concentration in *Colletes* host plant floral pollen.

Pollen	Polypeptide concentration	
	ug/mg	%
Aster tripolium	139,03	13,90
Calluna vulgaris	133,65	13,37
Hedera helix	135,56	13,56

The fresh weight of pollen loaves of *C. halophilus* is less than of *C. hederae*. In contrast the dry weight of pollen loaves of *C. halophilus* is higher than of *C. hederae*. Pollen loaves of *C. succinctus* are 50-60% lighter than the pollen loaves of the other two species.

The polypeptide concentration is similar in pollen loaves of *C. halophilus* and *C. hederae*. However in pollen loaves of *C. halophilus* polypeptide quantity is higher, due to the higher dry weight. In contrast pollen loaves of *C. hederae* contain more nectar due to the fact that their fresh weight is much higher than their dry (i.e. pollen) weight. Pollen loaves of *C. succinctus* have a 45-60% higher polypeptide concentration than pollen loaves of the other two species. Due to their small weight and high polypeptide concentration, it is supposed that they contain less nectar than pollen loaves of *C. halophilus* and *C. hederae*.

Between the three *Colletes* species there are both, different female investment (i.e. weight of the pollen loaves) and different female provisioning strategies (i.e. pollen-nectar mixture). In addition they may adopt pollen provision for their offspring to their own size. The exception is for female pollen loaves of *C. halophilus*. Although females of *C. halophilus* are a smaller than females of *C. hederae*, they make the same investment (i.e. weight of the pollen loaves) for their female pollen loaves. Females of *C. hederae* make higher dilution of the pollen. Females of *C. succinctus* are more than 30% smaller than females of the other species. They compensate their small investment capability (i.e. small pollen loaves) with reduction of pollen dilution to achieve an adequate high total polypeptide quantity of pollen loaves for their offspring.

5.3. Impact of pollen loaf quantity and quality on imago size

5.3.1. Intraspecific

There is a positive correlation between pollen loaf weight and adult sexual size dimorphism. Female pollen loaves as well as female adults are larger than male pollen loaves and male adults respectively.

There is a positive correlation between total polypeptide content of pollen loaves and adult sexual size dimorphism. Female brood cells contain a higher polypeptide quantity than male pollen loaves and female adults are larger than male adults.

Larvae of *Bombus terrestris* become larger, when they are provided with a polypeptide-rich diet (VANDERPLANCK et al., in press). Larvae of *Lasioglossum zephyrum* stay smaller when they are provided with a protein-poor diet (ROULSTON & CANE 2002). That conforms to the results for of the present study. Female offspring is provided with a higher polypeptide quantity than male offspring and thus becomes larger.

5.3.2. Interspecific

There is a positive correlation between pollen loaf weight and adult size. The exception are female pollen loaves of *C. halophilus*. Their fresh weight is similar to that of *C. hederae*

pollen loaves and their dry weight is even much higher. However female adults of *C. halophilus* are much smaller than females of *C. hederae*.

There is no correlation between the total polypeptide content of larval provision and the adult size. Female pollen loaves of *C. halophilus* contain 25% more polypeptides than female pollen loaves of *C. hederae*. However adult females of *C. halophilus* are 12% smaller. The same can be seen by regarding males of *C. halophilus*. Their pollen loaves contain 20% more polypeptides, but their adult size is 16% smaller than in *C. hederae*.

In former studies it was reported that body size is related to total protein which is consumed by the offspring (ROULSTON & CANE 2002, VANDERPLANCK et al., in press).

5.4. Discrepancy in pollen loaf and adult size relation of *C. halophilus*

The small body size of especially female adults of *C. halophilus* can not be explained by deficiency in provision quantity or quality. *C. halophilus* females mainly forage on Asteraceae (MÜLLER & KUHLMANN 2008). In general Asteraceae have a low protein content of 11.7-34.4% (ROULSTON et al. 2000). That is of course a limiting factor for many bee species. However the host plants of *C. hederae* and *C. succinctus* also contain little protein (approximately 13.5%, VANDERPLANCK et al., submitted). Furthermore protein quantity of pollen loaves available for offspring of *C. halophilus* is higher than for the larger species *C. hederae* (present study). Within the genus *Colletes* Asteraceae is the most frequent host plant family (MÜLLER & KUHLMANN 2008). However Asteraceae pollen were found in high amounts in oligolectic species, they occur only marginally in diets of polylectic species. Pollen of Asteraceae was reported to contain unfavorable or toxic substances which may delay or prevent larval development (MÜLLER & KUHLMANN 2008, PRAZ et al. 2008). Furthermore it was found that Asteraceae contain very low amounts of essential amino acids like arginine (all members of Asteroideae) and phenylalanine (PRAZ et al. 2008). The preferred pollen host of *C. halophilus*, *Aster tripolium*, belongs to the Asteroidea. Probably the deficient in amino acids and existence of toxic substances leads to the small size of females of *C. halophilus*.

5.5. Future studies

This study presents answers to the question about adaptation of female provision behavior to pollen quality and of the impact of pollen quality on bee size. However, due to the unexpected facts that too early collected brood cells may only contain male eggs or larvae, and that too late collected brood cells already contain large larvae, the study lacks comprehensive sampling of material. Further studies should be carried out to achieve additional sampling to evaluate male and female brood cells of *C. hederae* and to analyze the weight of pollen loaves of *C. succinctus*. It is crutial to collect the brood cells at the right time. Furthermore the eggs or larvae of the collected brood cells should be removed and put into a deep-freezer of -80°C as fast as possible to avoid the death of the specimens before the analysis of flow cytometry.

Future studies with a biological perspective should include analyses of further chemical compounds (i.e. amino acids, alkaloids, sterols) of the pollen loaves. Experiences of egg transfer to different pollen loaves between the sibling species could give an insight in the phylogenetic limitation of host plant specialization. With regard to the female investment it would be very interesting to observe the foraging time females need to provide one brood cell with pollen. Further studies may also explain the restricted distribution of *C. halophilus*.

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7. References

ALBANS K.R., APLIN R.T., BREHCIST J., MOORE J.F., O'TOOLE C. 1980. Dufour's gland and its role in secretion of nest cell lining in bees of the genus *Colletes* (Hymenoptera: Colletidae). Journal of Chemical Ecology, Vol. 6, Issue 3, pp. 549-564.

ALMEIDA E.A.B. 2008. Colletidae nesting biology (Hymenoptera: Apoidea). Apidologie, Vol. 39, pp. 16-29.

ALMEIDA E.A.B., DANFORTH B.N. 2009. Phylogeny of colletid bees (Hymenoptera: Colletidae) inferred from four nuclear genes. Molecular Phylogenetics and Evolution, Vol. 50, pp.290-390.

ALMEIDA E.A.B., PIE M.R., BRADY S.G., DANFORTH B.N. 2012. Biogeography and diversification of colletid bees (Hymenoptera: Colletidae): emerging patterns from the southern end of the world. J. Biogeogr. Vol. 39, pp. 526-544.

BARTLETT M. 1937. Properties of sufficiency and statistical tests. In: Proceedings of the Royal Statistical Society Series A. 160, pp. 268–282.

BELISLE R.A., TURNER I.G., ANSELL M.P. 2011. Evidence of biocomposite structure in *Colletes halophilus* nest material. J Mater Sci Vol.46, pp.6154-6157.

BERG J.M., TYMOCZKO J.L., STRYER L. 2002. Biochemistry. W.H. Freeman, 5th edition.

BISCHOFF I., ECKELT E., KUHLMANN M. 2005. On the Biology of the Ivy-Bee *Colletes hedereae* Schmidt & Westrich, 1993 (Hymenoptera, Apidae). Bonner zoologische Beiträge, Vol.53 1/2, pp. 27-36.

BOSCH J., VICENS N. 2002. Body size as an estimator of production costs in solitary bee. Ecological Entomology, Vol. 27, pp. 129-137.

BRADY S.G., DANFORTH B.N. 2004. Recent intron gain in elongation factor-1_ (EF-1_) of colletid bees (Hymenoptera: Colletidae). Mol. Biol. Evol. 21 (4), 691–696.

BUCHMANN S. L. 1986. Vibratile pollination in Solarium and Lycopersicon: a look at pollen chemistry. In: D'Arcy W. G. (Ed.) Solanaceae: Biology and Systematics. Columbia Univ. Press, NY, pp. 237-252.

CAMPBELL N.A., REECE J.B. 2004. Biologie. Pearson Studium.

CANE J.H. 2008. Bees (Hymenoptera: Apoidea: Apiformes). Encyclopedia of Entomology. Vol. 2, pp. 419-434.

CASTELLANOS M. C., WILSON P., KELLER S. K., WOLFE A. D., THOMSON J. D. 2006. Anther Evolution: Pollen Presentation Strategies, When Pollinators Differ. The American Naturalist, Vol. 167, No. 2, pp. 288–296.

COOK J.M., CROZIER R.H. 1995. Sex determination and population biology in the hymenoptera. Trands Ecol. Evol., Vol. 10(7), pp.281-6.

DANFORTH B.N., FANG J., SIPES S. 2006a. Analysis of family-level relationships in bees (Hymenoptera: Apiformes) using 28S and two previously unexplored nuclear genes: CAD and RNA polymerase II. Molecular Phylogenetics and Evolution, Vol. 39, pp. 358-372.

DANFORTH B.N., SIPES S., FANG J., BRADY S.G. 2006b. The history of early bee diversification based on five genes plus morphology. PNAS. Vol. 103, no. 41. pp. 15118-15123.

DANFORTH B.N., CARDINAL S., PRAZ C., ALMEIDA E.A.B., MICHEZ D. 2013. The Impact of Molecular Data on Our Understanding of Bee Phylogeny and Evolution. Annu. Rev. Entomol. Vol. 58, pp. 57-78.

DINAN L., SAVCHENKO T., WHITING P. 2001. On the distribution of phytoecdysteroids in plants. Cell. Mol. Life Sci. 58, pp. 1121-1132.

DÖTTERL S., FÜSSEL U., JÜRGENS A., AAS G. 2005. 1,4-Dimethoxybenzene, a floral scent compound in willows that attracts an oligolectic bee. Journal of Chemical Ecology, Vol. 31, No. 12, pp. 2993-2998.

DÖTTERL S., VEREECKEN N. J. 2010. The chemical ecology and evolution of bee-flower interactions: a review and perspectives. Can. J. Zool. 88: 668-697.

ENGEL M.S. 2001. Monograph of the Baltic Amber bees and Evolution of the Aoidea (Hymenoptera). Bulletin of the American Museum of Natural History. Number 259.

HEFETZ A., Fales H.M., Batra S.W.T. 1979. Natural polyesters: Dufour's gland macrolytic lactones from brood cell laminesters in *Colletes* bee. Science, Vol. 204, pp. 415-417.

KIRBY, W. 1802. Monographia Apum Angliae, or, An Attempt to Divide into their Natural Genera and Families, such Species of the Linnean Genus *Apis,* as have been Discovered in England. [2 vols.] Ipswich: White.

KNOWLES A. 2011. Nest site preference of *Colletes halophilus* in East Anglia.

KUHLMANN M. 2000. Katalog der paläarktischen Arten der Bienengattung *Colletes* Latr., mit Lectotypenfestlegungen, neuer Synonymie und der Beschreibung von zwei neuen Arten (Hymenoptera: Apidae: Colletinae). Linzer biol. Beitr. Vol. 32/1, pp. 155-193.

KUHLMANN M. 2003. Zur Kenntnis paläarktischer Bienen der Gattung *Colletes* LATR. mit Beschreibung neuer Arten (Hymenoptera: Apidae: Colletinae). Linzer biol. Beitr. 35/2, pp. 723-746.

KUHLMANN M., ELSE G.R., DAWSON A., QUICKE D.L.J. 2007. Molecular, biogeographical and phenological evidence for the existence of three western European sibling species in the *Colletes succinctus* group (Hymenoptera: Apidae). Organisms, Diversity & Evolution, Vol.7, pp. 155-165.

KUHLMANN M., ALMEIDA E.A.B., LAURENCE N., QUICKE D.L.J. 2009. Molecular phylogeny and historical biogeography of the bee genus *Colletes* Latreille, 1802 (Hymenoptera: Apiformes: Colletidae), based on mitochondrial COI and nuclear 28S sequence data. Insect Systematics & Evolution, Vol. 40, pp. 291-318.

KUHLMANN M. 2011. Checklist of the Species of the Bee Genus *Colletes* Latreille 1802. NHM UK. www.nhm.ac.uk.

KUHLMANN M. 2011. Checklist of the Palaearctic and Oriental Species of *Colletes* Latr. UMH UK. www.nhm.ac.uk.

MICHENER C.D. 1944. Comperative external morphology, phylogeny, and a classification of the bees (Hymenoptera). Bulletin of the American Museum of Natural History. Vol. 82, art. 6.

MICHENER C.D. 1964. Evolution of the nests of bees. Am. Zoologist, Vol. 4, pp. 227-239.

MICHENER C.D. 2000. The Bees of the World. Baltimore MD: Johns Hopkins Univ. Press.

MICHENER C.D. 2007. The Bees of the World. Baltimore MD: Johns Hopkins Univ. Press.

MICHEZ D., PATINY S., RASMONT P., TIMMERMANN K., VEREECKEN N.J. 2008. Phylogeny and hostplant evolution in Melittidae *s.l.* (Hymenoptera: Apoidea). Apidologie 39, pp. 146-162.

MÜLLER A. 1995. Morphological Specializations in Central European Bees for the Uptake of Pollen from Flowers with Anthers Hidden in Narrow Corolla Tubes (Hymenoptera: Apoidea). Entomol Gener 20(1/2): 043-057.

MÜLLER A. 1996. Host-Plant specialization in Western Palearctic Anthidine bees (Hymenoptera: Apoidea: Megachilidae). Ecological Monographs, 66(2), pp. 235-257.

MÜLLER A., BANSAC N. 2004. A specialized pollen-harvesting device in western palaearctic bees of the genus *Megachile* (Hymenoptera, Apoidea, Megachilidae). Apidologie 35, pp. 329-337.

MÜLLER A. 2006. Unusual host plant of *Hoplitis pici*, a bee with hooked bristles on its mouthparts (Hymenoptera: Megachilidae: Osmiini). Eur. J. Entomol., 103: 497-500.

MÜLLER A., DIENER S., SCHNYDER S., STUTZ K., SEDIVY C., DORN S. 2006. Quantitative pollen requirements of solitary bees: Implications for bee conservation and the evolution of bee-flower relationships. Biological Conversation 130, pp. 604-615.

MÜLLER A. 2008. A specialized pollen-harvesting device in European bees of the genus *Tetraloniella* (Hymenoptera, Apidae, Eucerini). Linzer boil. Beitr., 40/1, pp. 881-884.

MÜLLER A., KUHLMANN M. 2008. Pollen hosts of western palaearctic bees of the genus *Colletes* (Hymenoptera: Colletidae): the Asteraceae paradox. Biological Journal of the Linnean Society, Vol. 95, pp.719-733.

NATION J.L. 2002. Insect Physiology and Biochemistry. CRC Press LLC.

POUVREAU A. 2004. Les insectes pollinisateurs. Delachaux et Niestlé, Paris, 192p.

PRAZ C. J., MÜLLER A., DORN S. 2008. Specialized bees fail to develop on non-host pollen: do plants chemically protect their pollen? Ecology, 89(3), pp. 795-804.

RASMONT P., REGALI A., INGS T. C., LOGNAY G., BAUDART E., MARLIER M., DELCARTE E., VIVILLE P., MAROT C., FALMAGNE P., VERHAEGHE J.-C., CHITTKA L. 2005. Analysis of Pollen and Nectar of Arbutus unedo as a Food Source for *Bombus terrestris* (Hymenoptera: Apidae). J. Econ. Entomol. 98(3), pp. 656-663.

RHARRABE K., SAYAH F., LAFONT R. 2010. Dietary effects of four phytoecdysteroids on growth and development of the Indian meal moth, *Plodia interpunctella*. Journal of Insect Science, Vol. 10, Article 13.

RIBEIRO M.F., VELTHUIS H.H.W., DUCHATEAU M.J., VAN DER TWEEL I. 1999. Feeding frequency and caste differentiation in *Bombus terrestris* larvae. Insectes soc., Vol. 46, pp 306-314.

ROOIJAKKERS E.F., SOMMEIJER M.J. 2009. Gender specific Brood Cells in the solitary Bee *Colletes halophilus* (Hymenoptera; Colletidae). J Insect Behav. Vol. 22, pp. 492-500.

ROULSTON T.H., CANE J.H., BUCHMANN S.L. 2000. What governs protein content of pollen: pollinator preferences, pollen-pistil interactions, or phylogeny? Ecological Monographs, Vol 70(4), pp.617-643.

ROULSTON T.H., CANE J.H. 2002. The effect of pollen protein concentration on body size in the sweat bee *Lasioglossum zephyrum* (Hymenoptera: Apiformes). Evolutionary Ecology. Vol. 16, pp. 49-65.

SEDIVY C., MÜLLER A., DORN S. 2011. Closely related pollen generalist bees differ in their ability to develop on the same pollen diet: evidence for physiological adaptations to digest pollen. British Ecological Society, Functional Ecology 2011, doi: 10.1111/j.1365-2435.2010.01828.x.

SCHLINDWEIN C., WITTMANN D., MARTINS C. F., HAMM A., SIQUEIRA J. A., SCHIFFLER D., MACHADO I. C. 2005. Pollination of *Campanula rapunculus* L. (Campanulaceae): How much pollen flows into pollination and into reproduction of oligolectic pollinators? Plant Systematics and Evolution, Vol. 250, No. 3-4, pp. 147-156.

SCHMIDT J.O., BUCHMANN S.L. 1985. Pollen digestion and nitrogen utilization by *Apis mellifera* L. (Hymenoptera: Apidae). Comparative Biochemistry and Physiology, Part A: Physiology, Vol. 82, Issue 3, pp. 499-503.

SCHMIDT K., WESTRICH P. 1993. *Colletes hedereae* n. sp., eine bisher unbekannte, auf Efeu (Hedera) spezialisierte Bienenart (Hamenoptera: Apoidea). Entomol. Z. Vol. 103, pp. 89-112.

SHREEVES G., FIELD J. 2008. Parental care and sexual size dimorphism in wasps and bees. Behav. Ecol. Sociobiol., Vol. 62, pp. 843-852.

SOMMEIJER M.J., ROOIJAKKERS E.F., JACOBUSSE C., KERKVLIET J.D. 2009. Larval food composition and food plants of the solitary bee *Colletes halophilus* (Hymenoptera: Colletidae). Journal of Apicultural Research and Bee World, Vol. 48(3), pp. 149-155.

STEARNS S.C. 1977. The Evolution of Life history traits: A critique of the theory and a review of the data. Ann. Rev. Ecol. Syst. Vol. 8, pp. 145-71.

STEPHEN W.P., BOHART G.E., TORCHIO P.F. 1969. The Biology and external Morphology of Bees. agricultural Experiment Station. Oregon State University, Corvallis, Oregon.

STUBBLEFIELD J.W., SEGER J. 1994. Sexual dimorphism in the Hymenoptera. In: The differences between the Sexes. Short R.V., Balaban E. Cambridge University press.

THOMSON J. 2003. When Is It mutualism? Am. Nat., Vol. 162, pp. S1-S9.

TOLLSTEN L., KNUDSEN J. T. 1992. Floral scent in dioecious *Salix* (Salicaceae)-a cue determining the pollination system? Pl. Syst. Evol. 182: 229 – 237.

TORCHIO P.F., TROSTLE G.E., BURDICK D.J. 1988. The nesting biology of *Colletes kincaidii* Cockerell (Hymenoptera: Colletidae) and development of its immature forms. Ann. Entomol. Soc. Am., Vol. 81, pp. 605-625.

VANDERPLANCK M., LEROY B., WATHELET B., WATTIEZ R., MICHEZ D. Submitted. Standardised protocol to evaluate pollen proteins as bee food source. Edited for the journal Apidologie.

VANDERPLANCK M., MOERMAN R., RASMONT P., LOGNAY G., SUCHAI S., WATHELET B., WATTIEZ R., MICHEZ D. In press. Pollen nutritional content: what matters for polylectic bees?

VEREECKEN N.J., TOFFIN E., MICHEZ D. 2006. Observations relatives à la biologie et la nidification de quelques abeilles sauvages psammophiles d'intérêt en Wallonie. 2. Observations estivales et automnales. Parcs et Réserves, Vol.61, n°4.

VEREECKEN N.J., SCHWENNINGER H., GOGALA A., PROSI R., ROBERTS S.P.M. 2009. Mise à jour de la distribution de l'abeille du lierre, *Colletes hederae* SCHMIDT & WESTRICH (Hymenoptera, Colletidae) en Europe. Osmia, n°3.

VERHOEFF 1943. Opmerkingen over Hymenoptera ÿculeate. Verslag van de Zes-en-zeventigste Wintervergadering der Nederlandse Entomologische Vereniging, March 6.

WCISLO W.T. 2000. Environmental hierarchy, behavioral contexts, and social evolution in insects. Oecologia Brasiliensis. Pp. 49-84.

WCISLO W. T., CANE J. H. 1996. Floral resource utilization by solitary bees (Hymenoptera: Apoidea) and exploitation of their stored foods by natural enemies. Annu. Rev. Entomol, 41, pp. 257-286.

WEINER C.N., HILPERT A., WERNER M., LINSENMAIR K.E., BLÜTHGEN N. 2010. Pollen amino acids and flower specialisation in solitary bees. Apidology, Vol. 41, pp. 476-487.

WESTERKAMP C., CLAßEN-BOCKHOFF R. 2007. Bilabiate Flowers: The Ultimate Response to Bees? Annals of Botany 100: 361-374.

WESTRICH P. 2008. Flexibles Pollensammelverhalten der ansonsten streng oligolektischen Seidenbiene *Colletes hederae* Schmitd & Westrich (Hymenoptera: Apidae). Eucera, Vol. 1(2), pp. 17-32.

WIGHTMAN J.A. & ROGERS V.M. 1978. Growth, energy and nitrogen budgets and efficiencies of the growing larvae of *Megachile pacifica* (Panzer) (Hymenoptera: Megachilidae). Oecologia, Vol. 36, Issue 2, pp. 245-257.

Appendix 1

Adapted protocol for polypeptide quantification of pollen loaves

A) Preparation of samples

Weigh 5mg of a lyophilized pollen loaf in an Eppendorf tube.

B) Polypeptide extraction

Add 500µl of extraction buffer and heat for a minimum of 5 minutes in a water bath with temperature of 90°C. Place the samples immediately on ice.

Add 500µl of phenol and incubate for 10 minutes under agitation. Place in the centrifuge for 10 minutes with a speed of 13.2 rpm.

Transfer 400µl of the upper phenolic phase into a new tube.

Add 1.6ml of iced ammonium acetate (in 100% methanol) and let incubate overnight at -20°C.

C) Purification of the polypeptides

Place the tubes in the centrifuge for 10 minutes with a speed of 13.2 rpm. Remove the supernatant without contacting the polypeptide precipitate.

Add 1.5ml of methanol 100%, vortex and treat with ultrasonic waves until the whole precipitate is in solution again. Centrifuge for 10 minutes with a speed of 13.2 rpm. Remove the supernatant without contacting the polypeptide precipitate.

Add 1.5ml of methanol 100% once again, vortex and treat with ultrasonic waves until the whole precipitate is in solution again. Place in the centrifuge for 10 minutes with a speed of 13.2 rpm. Remove the supernatant without contacting the polypeptide precipitate.

Add 1.5ml of acetone 80%, vortex and treat with ultrasonic waves until the whole precipitate is in solution again. Place in the centrifuge for 10 minutes with a speed of 13.2 rpm. Remove the supernatant without contacting the polypeptide precipitate.

Do the purification with acetone 80% once again, if the precipitate wasn't totally in solution. Remove the supernatant without contacting the polypeptide precipitate and let it dry under an exhaust hood.

Add 0.5ml or 1ml of a guanidine-hydrochloride-buffer (depends on the size of the precipitate) and vortex until the whole precipitate is in solution again. Place the samples on ice.

D) Polypeptide quantification

Prepare a calibration standard of 8 different concentrations of BSA in guanidine-hydrochloride-buffer: 2000, 1500, 1000, 750, 500, 250, 125 and 25µg/ml.

Place the standard series in the lines A, B, F and G of a microwell plate (10µl per well). Start with the 'blank' (only guanidine-hydrochloride-buffer, polypeptide concentration=0 µg/ml). Place triplicates of the samples between the standard rows (10µl per well). Follow the scheme illustrated in Table 1.

Prepare the coloration solvent: substance A and B of BCA Protein Assay Kit (Pierce, Thermo Scientific) in a mixture of 50:1 ml. Place 200µl of the solvent into each well. Tap the plate carefully on the working surface and incubate for 30 minutes at 37°C under agitation.

Scan the absorption at a wavelength of 570nm at three different time intervals: t0 (immediately after incubation), t5 (after five minutes) and t10 (after 10 minutes).

Table 13. Scheme showing the charging of a microwell plate for analysis of polypeptides. Grey shaded rows: four lines of the standard series (denoted as concentration of BSA in µg/ml). X1-X12: samples placed as triplicates.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A		blank	25	125	250	500	750	1000	1500	2000		
B		blank	25	125	250	500	750	1000	1500	2000		
C	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12
D	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12
E	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12
F		blank	25	125	250	500	750	1000	1500	2000		
G		blank	25	125	250	500	750	1000	1500	2000		
H												

Appendix 2

Frequencies of fresh weight of pollen loaves / brood cells