FACULTY OF BIOSCIENCE ENGINEERING

UNCOVERING DIFFERENT STRESS-RELATED ADAPTATIONS BETWEEN AGRICULTURAL AND NATURAL BUMBLEBEES

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ACKNOWLEDGMENT (ENGLISH)

It goes without saying that this bumblebee thesis, going from manual fieldwork in the Flemish meadows and forests to scientific research in the lab of Ghent University, was a very fascinating experience! Above all, it was an enriching growth process with some personal highlights, a unique experience and wonderful ending of the Bioengineering study programme: from the first tentative steps in catching and identifying the different bumblebee species and eliminating the "bumblebee look-a-likes" to a quick recognition of *Bombus pascuorum*; from finding the right course of action when working with these delicate, yet very active insects (getting a bumblebee into a mini tube is quite a challenge), to a smooth and almost natural routine; from the knowledge gained from the literature study to the challenges of real lab work, with an ever-growing curiosity about the outcome of our own experiments.

The past five years have been incredibly enriching and intensive, with inspiring lectures, surprising practical lessons and countless block sessions. In short, learning, trying, correcting and, above all, never giving up.

I still remember the first day when everything started, the meeting day at the faculty with all 1st year students, still very ignorant of what was to come. Just 18 years old, with a bit of stress and without knowing anyone, heading to the Coupure campus and the Oehoe room. It was a very exciting start, even the best anyone could wish for, followed by lots of new friends, funny moments and fantastic memories. Even though everyone is going their separate ways soon, I was lucky to make friends for life and as I conclude this education, I say in confidence: "see you soon" and not "farewell".

The atmosphere and communication at the faculty was very encouraging to keep on going and giving everything until the end. So I would like to thank all fellow students, PhD students and professors very much, because staying hopeful and optimistic is contagious and inspiring. Even if it was just a smile or "hello" in the corridor, it was nice to see how everyone supported each other and how everyone could be themselves on this fantastic campus.

In particular, I would like to thank my promotor, Dr. Kevin Maebe, for sharing his knowledge and experiences about the fascinating insect world, as well as for his sincere commitment and belief in his thesis students, for trusting us to work independently. Despite his busy schedule, he took the role as tutor and promotor entirely to heart. There was always time to discuss proposals and seek answers to (the many) questions. Especially his positive and constructive feedback and encouragement to take on additional challenges will always stay with me. I found it a privilege to have worked with Dr. Maebe.

Of course, I would also like to thank my mum and dad, because after a tiring day in Ghent, being able to come home in a “warm nest” and recover from the day always makes for great memories. Their interest, empathy and unconditional support in every challenge I face, give me strong wings!

I am grateful to my remarkable Omi, because she taught me from an early age to look at the world with an open mind and, above all, to pause and look with wonder. After all, life depends on little things that we take for granted, but are not. The bumblebee is one of these little things.

I must not forget my two animal friends. Snowy, my bunny who patiently studied by my side and never missed a course for five years. He is and always will be the perfect study companion! And Jochem, my enthusiastic Appaloosa pony who makes my life a lot more adventurous and helps me to relief the study stress.

Where my family and friends are concerned, my enthusiasm has for sure increased their attention to and knowledge of bumblebees. This spring, a wide variety of insect hotels were put up and opened. May the bees and bumblebees benefit from this.

In short, thank you to everyone who was with me this year. I am incredibly grateful and proud to have been part of this journey. What the future holds, is still a big question mark. However, I look forward to it with confidence thanks to this study. As long as you follow your feelings, show commitment and dare to try, new doors will open. I look forward to new challenges and new adventures. In the meantime, I try to "Bee" the change I want to see in the world!

DANKWOORD (NEDERLANDS)

Het spreekt voor zich dat deze hommelthesis, gaande van manueel veldwerk in de Vlaamse weilanden en bossen tot wetenschappelijk onderzoek in het labo van de UGent, een zeer leerrijke ervaring was! Het was bovenal een verrijkend groeiproces met enkele persoonlijke hoogtepunten, een unieke belevenis en prachtige afsluiter van de opleiding tot Bio-Ingenieur: van de eerste voorzichtige stappen bij het vangen en determineren van de verschillende hommelsoorten en het elimineren van de "hommel-look-a-likes" tot een snelle herkenning van de *Bombus pascuorum*; van het zoeken naar de juiste handelswijze bij het werken met deze delicate doch zeer bedrijvige insecten (een hommel in een minitube krijgen is een hele uitdaging), tot een soepele en haast vanzelfsprekende routine; van de opgedane kennis uit de literatuurstudie tot de uitdagingen van het labowerk, met een steeds groeiende nieuwsgierigheid naar de uitkomst van onze eigen experimenten.

De voorbije vijf jaren waren ontzettend verrijkend en intensief, met inspirerende colleges, verrassende praktijklessen en talloze afmattende bloksessies. Kortom bijleren, proberen, corrigeren en vooral nooit opgeven.

Nog altijd herinner ik me de eerste dag waarop alles begon, de ontmoetingsdag op de faculteit met alle 1^{e} bachelor studenten, nog heel onwetend over wat komen zou. Net 18 jaar, met een beetje gezonde stress en zonder iemand te kennen, op weg naar de Coupure campus en het Oehoe lokaal. Het was een zeer spannende start, maar wel de beste die iemand zich wensen kon, gevolgd door heel veel nieuwe vrienden, leuke en grappige momenten en fantastische herinneringen. Ook al gaat iedereen binnenkort zijn eigen weg, ik had het geluk hier vrienden voor het leven te mogen maken en zeg ze bij het afsluiten van deze opleiding welgemeend "tot binnenkort" en niet "vaarwel".

De sfeer en communicatie op de faculteit was zeer bemoedigend om verder te blijven doen en alles te geven tot het einde. Ik wil hierbij dan ook alle medestudenten, PhD studenten en proffen hartelijk danken, want samen hoopvol en optimistisch blijven, werkt aanstekelijk en inspirerend. Ook al was het maar een welgemeende glimlach of "hallo" in de gang, het was mooi om te zien hoe iedereen elkaar steunt en hoe iedereen zichzelf kan zijn op deze fantastische campus.

Ik wil in het bijzonder mijn promotor Dr. Kevin Maebe bedanken voor het delen van zijn kennis en ervaringen over de boeiende insectenwereld, alsook voor de oprechte betrokkenheid en het geloof in zijn thesis studenten, voor het vertrouwen om ons de kans te geven zelfstandig te werken. Ondanks zijn drukke agenda, nam hij de rol als tutor en promotor geheel ter harte. Er werd steeds tijd vrij gemaakt om voorstellen te bespreken en te zoeken naar antwoorden op (de vele) vragen. Vooral zijn positieve en constructieve feedback en aanmoedigingen om extra uitdagingen aan te gaan, zal me steeds bijblijven. Ik vond het een voorrecht om met Dr. Maebe te hebben mogen samenwerken.

Ik wil natuurlijk ook mijn mama en papa bedanken, want na een vermoeiende dag in Gent, zorgt het gezellig "thuis komen" in een warm nestje en even kunnen bijkomen van de dag altijd voor mooie herinneringen. Hun interesse, inleving en onvoorwaardelijke steun bij elke uitdaging die ik aanga, geven me sterke vleugels!

Mijn bijzondere Omi ben ik dankbaar omdat ze me van kleins af aan heeft geleerd om met een open blik naar de wereld te kijken en vooral om op tijd even stil te staan en te kijken met verwondering. Het leven hangt immers af van kleine dingen die we als vanzelfsprekend beschouwen, maar dat niet zijn. Laat de "hommel" daar nu één van zijn.

Ik mag ook mijn twee dierenvrienden niet vergeten. Snowy, mijn konijntje dat geduldig naast mijn zijde mee studeerde en vijf jaar lang geen cursus miste. Hij is en blijft de perfecte studiegenoot! En Jochem, mijn enthousiaste Appaloosa pony die mijn leven heel wat avontuurlijker maakt. Onze gezamenlijke ritjes zijn de ideale uitlaatklep om de studiestress te doen verdwijnen.

Voor mijn familie en al mijn vrienden geldt dat hun aandacht en kennis van hommels door mijn enthousiasme alvast is toegenomen. Dit voorjaar werd een grote variatie aan insectenhotelletjes geplaatst en geopend. Mogen de bijen en hommels er wel bij varen.

Kortom, dank u wel aan iedereen die er dit jaar bij was. Ik ben ontzettend dankbaar en trots om dit mee te mogen maken. Wat de toekomst brengt, is nog een groot vraagteken. Ik zie ze dankzij deze studie vol vertrouwen tegemoet. Zolang je maar je gevoel volgt, inzet toont en durft te proberen, zullen er deuren opengaan. Ik kijk uit naar nieuwe uitdagingen en avonturen. In tussentijd: I try to “Bee” the change I want to see in the world!

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Ghent, 8 June 2023

The promotor,

Dr. Kevin Maebe

The author,

Lieselotte Leus

SUMMARY (ENGLISH)

Bumblebees are vital to the climate and life on Earth. They pollinate flowers, crops and are essential to preserve nature. Their global decline is linked to various environmental and anthropogenic stressors.

For this thesis, 738 field collected *Bombus pascuorum* from agricultural (412) and natural (326) areas were used to uncover stress-related adaptations. Three main stressors were studied: pesticides (different Spinosad concentrations), malnutrition (reduced 25% sugar concentration) and temperature (thermal range in incubator). For pesticides and nutrition, survival time was monitored, whereas for the temperature experiment, critical thermal limits were determined.

Our results show that high Spinosad concentrations of 40 µg/mL, 4 µg/mL and 0.4 µg/mL are lethal and that a low concentration of 0.04 µg/mL seems harmless. Bumblebees from agricultural areas survive longer and may be better adapted to pesticides than individuals from natural areas, as was shown for the 4 µg/mL concentration. Bumblebees from both places were able to cope with malnutrition and have the same critical temperatures. The mean CT_{min} and CT_{max} are 0.6°C and 47.7°C, respectively.

The presence of *Apicystis* (40.4%), *Crithidia* (20.8%) and *Vairimorpha* (14.0%) was detected by qPCR after DNA extraction. An unexpected result was the presence of the conopid fly (12.1%). None of these pathogens influenced the outcome of the experiments. Relevant morphological parameters were also collected (mass, length radial cell right/left forewing, length of the six left middle leg compartments).

This study provides insight into some resilience factors that bumblebees develop in different environments (agricultural *versus* more natural) in order to survive.

Key words: Bumblebees, agricultural & natural areas, heat stress, pesticides, malnutrition, pathogens, phenotypic plasticity, adaptations

SAMENVATTING (NEDERLANDS)

Hommels zijn van vitaal belang voor het klimaat en het leven op Aarde. Ze bestuiven bloemen, gewassen en zijn essentieel voor het behoud van de natuur. Hun wereldwijde achteruitgang wordt in verband gebracht met verschillende omgevings- en antropogene stressfactoren.

Voor deze thesis werden 738 in het veld verzamelde *Bombus pascuorum* uit landbouw- (412) en natuurlijke (326) gebieden gebruikt om stress-gerelateerde aanpassingen te achterhalen. Drie belangrijke stressfactoren werden bestudeerd: pesticiden (verschillende Spinosad concentraties), ondervoeding (verlaagde suikerconcentratie 25%) en temperatuur (thermische grenzen in een incubator). Voor het pesticide en voedingsexperiment werd de levensduur opgevolgd, terwijl voor het temperatuurexperiment de kritische temperatuurlimieten werden bepaald.

Onze resultaten tonen aan dat hoge Spinosad-concentraties van 40 µg/mL, 4 µg/mL en 0,4 µg/mL dodelijk zijn en dat een lage concentratie van 0,04 µg/mL ongevaarlijk lijkt. Hommels uit landbouwgebieden overleven langer en zijn mogelijk beter aangepast aan pesticiden in vergelijking met hommels uit natuurgebieden, zoals werd vastgesteld bij de 4 µg/mL concentratie. Hommels van beide habitats konden even goed omgaan met een verminderde suikerconcentratie. De gemiddelde CT_{min} en CT_{max} waren gelijk voor beide habitats en bedroegen respectievelijk 0,6°C en 47,7°C.

De aanwezigheid van *Apicystis* (40,4%), *Crithidia* (20,8%) en *Vairimorpha* (14,0%) werd gedetecteerd met qPCR na DNA-extracties. De aanwezigheid van de blaaskopvlieg (12,1%) was onverwacht. Geen van deze ziekteverwekkers beïnvloedde de uitkomst van de experimenten. Relevante morfologische parameters werden verzameld (massa, lengte radiaalcel rechter/linker voorvleugel, lengte van zes compartimenten linker middenpoot).

Deze studie geeft inzicht in enkele aanpassingsfactoren die hommels ontwikkelen in verschillende omgevingen (landbouw *versus* meer natuurlijk) om te overleven.

Trefwoorden: Hommels, landbouw en natuurlijke gebieden, hittestress, pesticiden, ondervoeding, pathogenen, fenotypische plasticiteit, aanpassingen

LIST OF ABBREVIATIONS

ABPV	Acute bee paralysis virus
AIC	Akaike information criterion
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
B.	*Bombus*
BQCV	Black queen cell virus
CAP	Common Agricultural Policy
Cq	Quantification cycle
CT_{max}	Critical maximum temperature
CT_{min}	Critical minimum temperature
Df	Degrees of freedom
DNA	Deoxyribonucleic acid
DWV	Deformed wing virus
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EU	European Union
F & R primers	Forward and reverse primers
GABA	Gamma-amino-butyric acid
GLM	Generalised linear models
GLMM	Generalised linear mixed models
Hsp	Heat shock protein
IDT	Integrated DNA technologies
L	Agricultural area
LD50	Median lethal dose for 50% of individuals
LM	Linear models
LSV	Lake Sinai virus
Mb	Megabases
N	Natural area
P0 – 4	Pesticide concentrations 0 – 4
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RADseq	Restriction-site-Associated DNA sequencing
RNA	Ribonucleic acid
RNAseq	RNA sequencing
Rpm	Rounds per minute
S0 – 1	Sugar concentration 0 – 1
SBV	Sacbrood bee virus
χ^2	Chi-squared

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1. INTRODUCTION

The worldwide declining number of bumblebee species in the past decades cannot be ignored and people should be aware of the dangers this entails. Bumblebees are very important for the climate and life on earth of all animals, plants and humans. They pollinate flowers, crops and are essential to preserve nature.

Obtaining a good insight in the resilience factors which bumblebees have developed in order to adapt to and survive the changing environment and the increasing number of anthropogenic and natural stressors, is essential to protect their wellbeing.

Previous research has tested the effects of individual stressors on different bumblebee species, some studies focusing on commercial bumblebees, others on old museum specimens and recently collected specimens. Several candidate genes were identified and linked to possible adaptations of bumblebees to individual stressors (Hart et al., 2022). However, not much is known about the interactions and combined influence of multiple stressors.

In this thesis, the impact of multiple stress factors was tested and compared between field-collected bumblebees from agricultural and from more natural environments, focusing on one species "*Bombus pascuorum*". Pesticide, temperature and nutrition stress experiments were performed, the underlying presence of pathogens tested and several morphological parameters measured, to uncover whether there are noteworthy differences or adaptations related to improved responses or abilities to survive between bumblebees originating from different environments.

2. LITERATURE STUDY

2.1 BUMBLEBEES

2.1.1 Taxonomy

Worldwide, there are more than 250 bumblebee species (genus *Bombus*, part of the Apidae or bee family), most of which are native to the Northern hemisphere (Europe, North Africa, North America, Asia) and to a lesser extent to the Southern hemisphere (South America) (Wilson-Rich, 2016; Michez et al., 2019). Additionally, bumblebees were introduced in New Zealand and Tasmania (Schmid-Hempel et al., 2007).

"Bumblebee" and "humblebee" are both frequently used denominations, which relate to the "buzzing", "bumming" or "humming" sound these large flying insects make. The genus *Bombus* comes from Latin, which also means "buzzing" or "humming" (Michez et al., 2019). The "buzzing" differs by species and is in general size related. It can be low- (e.g. *B. hortorum*) or high-pitched (e.g. *B. sylvarum*). The sound is not only produced by the rapid motion of vibrating wings, but also by air passing over the membranes of the spiracles (little air holes) (Peeters et al., 2012).

In literature, insect taxonomy and classification have been under debate for years and are subject to reorganisation. Different qualification criteria, such as evolution, morphology, behaviour, molecular phylogenetics and more recently DNA (deoxyribonucleic acid) sequencing, result in alternative hierarchical trees, increasing the general need for a global taxonomy and an up-to-date system.

Table 2.1: *Classification of the bumblebee.*

Kingdom: Animalia
Phylum: Arthropoda
Class: Insecta
Subclass: Pterygota
Infraclass: Neoptera
Superorder: Holometabola
Order: Hymenoptera
Suborder: Apocrita
Infraorder: Aculeata
Superfamily: Apoidea
Family: Apidae
Subfamily: Apinae
Tribe: Bombini
Genus: *Bombus*
Species: *pascuorum*

Table 2.1 gives a classification of the bumblebee, based on the online database Catalogue of Life (COL) (Thomson et al., 2018).

Insecta are the largest (75%) and most species rich class in the animal kingdom. They belong to the phylum of **Arthropoda** which consists of the jointed-limbed invertebrates with an exoskeleton and a segmented body. Insects (Latin: *insectum,* meaning "divided into segments") differ from other arthropods by their tripartite segmented body (Laget et al., 2009).

The vast majority of insects have or had wings, a feature that was sometimes lost during historical evolution, which places them in the subclass **Pterygota** (Greek: *pteryx*, meaning "wings"). If the insect has or had (in ancestor species) a flexing mechanism enabling it to fold its wings over its back, it is classified in the infraclass **Neoptera**. If the wing buds develop inside the body during larval stage (Endopterygota), which is found in insects that go through a complete metamorphosis with a distinctive larval, pupal and adult stage, they belong to the superorder **Holometabola**, whereas Hemimetabola (Exopterygota) have an incomplete metamorphosis where the wings are already externally visible before the adult stage. The order of **Hymenoptera** (Greek: *Hymen*, meaning "membrane") is characterised by membranous transparent wings and includes ants, wasps and bees (including bumblebees).

Hymenoptera without a petiole, i.e. a strong constriction between the first and second abdominal segment ("wasp waist"), are classified under the Symphyta. Hymenoptera with a petiole are classified as **Apocrita** (Falk, 2015). Apocrita are further split into two infraorders: the Terebrantia/Parasitica of which the female insects have an ovipositor and the **Aculeata**, in which the female ovipositor has evolved into a stinger. The Aculeata are split in three superfamilies: Vespoidea (wasps, ants), Chysidoidea (ruby-tailed wasps) and **Apoidea** (including all bee species) (Falk, 2015; Michez et al., 2019).

The species documented in Europe that belong to the Apoidea, are divided in six families: the Andrenidae, Colletidae, Halictidae and Melittidae (families of short-tongued bees) and the **Apidae** and Megachilidae (families of long-tongued bees). The Apidae contain the subfamilies **Apinae**, Nomadinae (cleptoparasitic species) and Xylocopini (carpenter bees). The Apinae contain solitary tribes Anthophorini and Eucerini, but also eusocial tribes like the bumblebees and honeybees (Wilson-Rich, 2016; Michez et al., 2019).

The tribe **Bombini** only contains one living genus i.e. ***Bombus***. Other genera such as the *Calyptapis* and *Oligobombus* have become extinct (Wilson-Rich, 2016; Michez et al., 2019). The genus *Bombus* counts over 250 species worldwide with *B. pascuorum*, *B. lucorum* and ***B. terrestris*** being abundantly present in Europe and Belgium. In this thesis we focus on *B. pascuorum* or "common carder bee".

2.1.2 Determination of bumblebee species

Bumblebees generally have furry bodies with a wide variation in coloration. **Colour** is thus an easy feature to distinguish between bumblebee species. Three distinct groups are used, namely: the red tailed, white tailed and ginger/brown back bumblebees, as seen in ***Figure 2.1***. In Europe, the most famous species of each group is *B. lapidarius*, *B. terrestris* and *B. pascuorum* respectively (Rasmont & Iserbyt, 2022).

Red tailed bumblebees (e.g. *B. lapidarius*, *B. pratorum*, *B. ruderarius*) have a red tail, which covers up to 50% of their abdomen. It is easy to distinguish between the various red tailed species, as they have different yellow band patterns. These patterns can also be used to distinguish between male and female bumblebees of the same species (Bumblebee Conservation Trust, 2021; Rasmont & Iserbyt, 2022).

The white tailed bumblebees (e.g. *B. terrestris*, *B. lucorum*, *B. hortorum*) have a white tail. As with the red tailed bumblebees, the yellow band pattern differs for the different species and sexes.

The ginger/brown back bumblebees (e.g. *B. pascuorum*, *B. muscuorum* and *B. humilis)* have a hairy, fluffy back with a uniform tail. The species are therefore harder to distinguish (Falk, 2015). The determination is not always obvious, since the colour patterns can differ within the same species depending on the geographical region where they live (Villers & Schoonvaere, 2019).

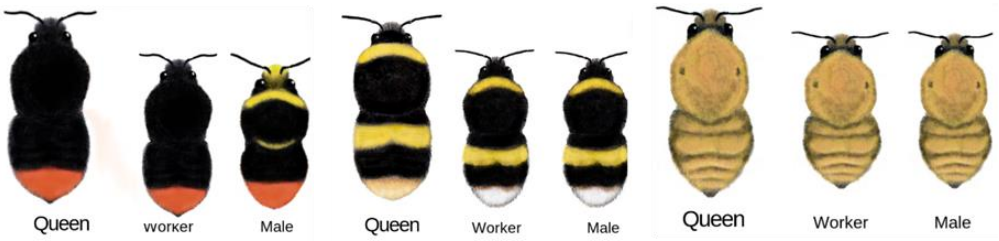

Figure 2.1: *Red tailed "*B. lapidarius*", white tailed "*B. terrestris*" and brown back "*B. pascuorum*" (Bumblebee Conservation Trust, 2021).*

The highly variable colour patterns serve various purposes such as camouflage and thermoregulation, designed to increase their chances of survival. Aposematic signals of bumblebees include their contrasting stripes (yellow/orange-black) in the fur, warning their predators that they are “not tasty” or poisonous, as well as some acoustic signals. When predators distinguish and recognise this look, they will avoid eating these individuals. The pattern and look can be mimicked by other species. When harmless species (e.g. flies) mimic harmful species, this is called Batesian mimicry. When harmful species (e.g. bumblebees) mimic each other, this is known as Müllerian (co)-mimicry (Jablonski et al., 2013; Rapti et al., 2014).

Bumblebees can also be classified according to their **behaviour**. Most bumblebees are eusocial, living in a highly organised colony. They have overlapping generations, a division of tasks and cooperative brood care (Libbrecht & Keller, 2015). Unlike these social bumblebees, who form colonies and make nests, other species such as the cuckoo bumblebees do not form colonies of their own. They invade other nests and let the workers of the original colony take care of their eggs.

2.1.3 Anatomy

The body of a bumblebee consists of three main parts (tagmata): the head (prosoma), the thorax (mesosoma) and the abdomen (metasoma). The basic anatomy is shown in ***Figure 2.2***.

The **head** has a pair of antennae, two compound eyes, three ocelli and a mouth.

The antennae (one on each side of the head) consist of three subparts: a scape, a pedicel and a flagellum, which consists of several segments. They act as sensors for touch, smell (pheromones), light, temperature, electric fields and chemicals. The antennae’s hairs contain pore plates that send information from the environment to sensory cell membranes with odour receptors for smell, gustatory receptors for taste and mechanoreceptors for touch. The latter enables them to sense movement of air particles, thus while not having physical “ears” they can “sense” hence “hear” airborne sound (Nadrowski et al., 2011; Michez et al., 2019).

Bumblebees have two big compound eyes on the sides of the head and three small ocelli positioned in a triangular shape on the top of the head. The compound eyes are composed of thousands of facets or ommatidia, giving a wide field of view in a mosaic way rather than one high resolution image. Bumblebees are trichromatic, with photoreceptors capturing short wavelengths, enabling them to see ultraviolet light (300-400 nm) that is invisible to humans, up to medium wavelengths of blue (400-500 nm) and longer wavelengths of green (500-600 nm). They cannot see red/orange wavelengths. Colour perception is important to find flowers with pollen and nectar. The ocelli are used to detect light (Dyer et al., 2011; Meyer-Rochow, 2019).

The mouth consists of multiple parts with different functions: labrum (front lip), mandibles (jaws), maxillae (two sheaths under mandibles that grasp and shape food), labial palps (taste sensors), proboscis (tube) with glossa, a tongue-like structure that protrudes from the proboscis to collect nectar.

The **thorax** contains two pairs of wings with wing muscles that control the wing movement, three pairs of air holes or spiracles which provide oxygen and three pairs of jointed legs (Michez et al., 2019).

On each side of the thorax, a large forewing and smaller hindwing couple together during flight with the help of hamuli, to create what seems as one wing (Basibuyuk & Quicke, 1997). The wings are composed of different cells and the fragile structure is clearly visible with the naked eye. A relatively

big radial cell at the side of each wing is linked to the average size of the bumblebee and used as a basic measurement to compare individuals (Owen, 1989; Michez et al., 2019). The wing beat is formed by the alternate contraction and lengthening of two powerful antagonistic muscles (the dorso-ventral and dorsal-longitudinal muscle), together with a complex hinge mechanism and resonating thorax. (Hedenström, 2014).

A bumblebee has six legs: two forelegs (pro), two middle legs (meso) and two hindlegs (meta), all composed of six segments: coxa, trochanter, femur, tibia, tarsus and metatarsus. The coxa connects with the thorax. Each pair of legs has different functions: The forelegs are used for cleaning the bumblebee's head. The middle legs have no essential function, but the bumblebees do hold these legs up in a defensive position when they are feeling threatened. In research on living specimens, one of the middle legs can be taken away for DNA extraction while the specimen survives. The hind legs of female bumblebees are used to collect and store pollen and nectar in the corbicula (pollen basket) during the flight back to the nest (Falk, 2015; Michez et al., 2019).

The **abdomen** contains the digestive, excretory and reproductive organs and has seven pairs of air holes. In female bumblebees the abdomen ends in the stinger, which is located underneath the rectum (Michez et al., 2019).

As stated before, bumblebees being arthropods possess a rigid exoskeleton, a multi-layered water-tight shield, that protects them from physical damage, injury and dehydration. It is made of the polysaccharide chitin and various proteins (Fabritius et al., 2011). During larval stage, the skin is shed periodically, but once hatched from its cocoon, the bumblebee and its exoskeleton stay the same size during its whole adult life.

Bumblebees have an open circulatory system. They have a coelom (body cavity) holding their internal organs immersed in haemolymph. The haemolymph is not confined to veins or arteries, but circulates freely and comes in contact with the tissues, transferring nutrients and wastes (Casem, 2016).

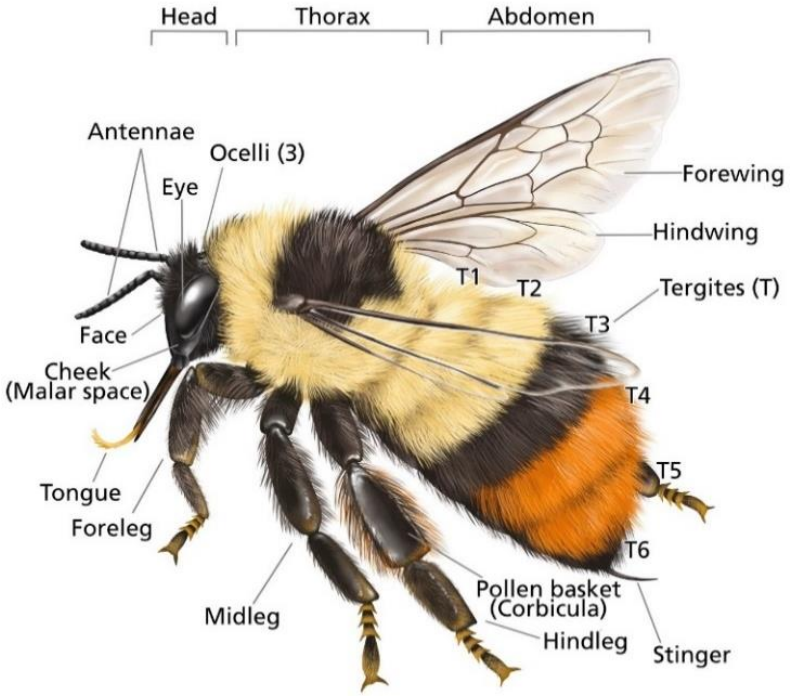

***Figure 2.2**: The general anatomy of bumblebees consisting of three major parts: head, thorax and abdomen (Mundy, n.d.).*

2.1.4 Drones, workers and queens

In bumblebees, sex is determined by one genetic locus. Males (or drones) are normally haploid and hemizygous for this locus as they only have one copy (E), females are diploid and heterozygous (D, E). In some cases, diploid drones (E,E) emerge which are homozygous (Wilson-Rich, 2016; EIS, 2021). To distinguish male drones from female workers or queens, some general characteristics can be looked at. Size can be used to differentiate the sex for most bumblebee species. The drones are typically bigger than the workers and when a female bumblebee is bigger than normal, this is probably a (future or daughter) queen.

Other distinguishing characteristics are:

- The abdomen of males, who cannot sting, has a blunt end, whereas that of females has a pointy end. A bumblebee has a smooth stinger and can therefore sting multiple times, whereas honeybees have a barbed stinger and die after stinging as they lose a part of their abdomen and venom gland. The venom of bumblebees contains bombolitin, whereas bee venom primarily contains pain inducing melittin making the sting more painful for humans (Rochette, 2006).
- If a bumblebee carries pollen on its hind legs, it will be female.
- Males have more hairs on their head than females.
- A female bumblebee's antennae are composed of 12 segments, a male's of 13 segments (Falk, 2015).
- Female bumblebees have six abdominal segments (tergites) whereas males have seven (Falk, 2015).
- Males are normally haploid and come from unfertilized eggs (no zygote, only "n" chromosomes), females are diploid and come from fertilized eggs (a zygote was formed and they have "2n" chromosomes). Females can be queens/daughter queens or workers.

2.1.5 Life cycle

Most bumblebee colonies have an annual life cycle. The flying period is different for various species. Some have relatively short or long cycles, with varying emergence and disappearance periods over different months. The production of one generation each year is called univoltinism. However, it is described that some species can produce multiple generations in one year (Skyrm et al., 2012). While the queen can live for approximately one year, the female workers have an average life span of four weeks and the average for males is "only" two weeks.

The life cycle begins when a queen awakens from hibernation at the beginning of spring. After having refuelled on early spring flowers, she looks for a good nesting site and starts laying eggs, which were fertilized in autumn of the year before. The nest, built with wax, is used to hold and protect the eggs and to store food. Bumblebee nests can be located underground or at hidden, covered and protected places, in an old barrel or compost site (Falk, 2015).

The queen broods the eggs and will not leave the nest after the first batch of workers has hatched. The evolution from eggs into adult bumblebees takes around four weeks. After four days, the eggs hatch into blind, legless larvae that look like maggots which will continuously grow and shed three times in

14 days, before pupating into a cocoon. The pupas will develop into mature bumblebees after another 14 days. All the queen's eggs that hatch in the first months of spring will normally be workers (all female) (Wilson-Rich, 2016).

The workers can be divided into two groups, i.e. the nesters and foraging bumblebees. The nesters stay in the nest to protect the colony, take care of the eggs and the larvae, while the foraging bumblebees will go outside the colony to gather pollen and nectar. After some months, female workers can start laying unfertilized eggs producing drones. However, this is not tolerated by the queen, who will eat and replace the eggs. From the new eggs produced by the queen, drones and future queens (gynes) are born. The drones' sole task is to fertilize the new queens, after which they die. This annual cycle ends in the fall after mating, when the new fertilized (daughter) queens search for a good spot to hibernate. The queen stores the sperm in the spermatheca, allowing her to control the fertilization of the eggs next spring (Falk, 2015; Michez et al., 2019; Timberlake, 2019)

The evolutionary stage of a colony at a certain point in time can be evaluated by looking if there are a lot of males/females present and how the nest evolves. The workers are present in spring and beginning of summer. During the following summer months, more drones will be born together with more future queens. In ***Figure 2.3***, the yearly colony life cycle of bumblebees is illustrated.

Figure 2.3*: One year life cycle of a bumblebee colony. The queen emerges from hibernation in early spring, refuels on pollen and nectar of spring flowers and starts a nest. She produces, fertilises and incubates a first batch of eggs, producing (female) workers. The colony grows and more workers are produced to help with the offspring with a peak in summer. By the end of summer, drones (male) and daughter queens (female) are produced. The old queen dies and the daughter queens mate, after which they look for a place to hibernate. The drones and workers do not hibernate and do not survive. Figure based on Timberlake (2019).*

2.1.6 Nutrition

Bumblebees are part of the Apidae family who need pollen and nectar during their whole life cycle in order to survive and are thus flower dependent. Nectar, rich in sugar, is used as energy source by adults. Pollen, being a high protein source, is collected to feed the larvae and is eaten by newly emerged daughter queens to develop the ovaries (Tanaka et al., 2019).

Their annual life cycle has synchronized their phenology with the availability of food resources. Consequently, the active months of bumblebees are the months when their favourite flowers will be flowering. Their diet and daily nutrition intake varies depending on the habitat, location of their nest and weather conditions. Unlike some bees, which depend on one (monolectic) or a few (oligolectic) plant species, most bumblebees are not plant specific (polylectic) and can be found visiting a broad range of flowers (Falk, 2015; Michez et al., 2019).

In general, bumblebees have a preference for four plant families: Fabaceae (e.g. *Trifolium*, *Medicago sativa*), Lamiaceae (e.g. *Lamium purpureum*), Boraginaceae (e.g. *Symphytum*) and Scrophulariaceae (e.g. *Syringa vulgaris*). Depending on the host plant, pollen have different nutritional values, containing some essential amino acids that bumblebees need as they cannot produce these themselves (Folschweiller et al., 2020).

The morphology of the flowers of the host plant also plays a decisive role in the preferences of bumblebees. Bumblebees with a long tongue can eat from flowers which are hard to reach for other species with a shorter tongue. The species with a shorter tongue visit flowers where the food is easier to reach or they bite their way into the flower, called nectar robbing. Difference in tongue length can lead to resource specialisation of bumblebees (Irwin & Maloof, 2002; Falk, 2015).

According to the way bumblebees store the flower pollen to feed their larvae, they can be divided into pocket makers or pollen storers. For pocket makers, the larvae can feed on a shared pollen clump that is continuously refilled, whereas for the pollen storers, pollen are stored in wax cells away from the larvae who are actively fed by the workers. In most cases, bumblebees with long tongues are pocket makers and bumblebees with short tongues are pollen storers. *B. pascuorum* is an example of a pocket maker, while *B. lapidarius* is a pollen storer (Peeters et al., 2012; Falk, 2015).

Bumblebees collect and store pollen and nectar only for a limited time. As the whole colony dies before winter, except for the hibernating queens, they do not need to store food for winter like honeybees. The place where the small quantities of nectar are stored, is referred to as “honey pots” and is characterised by a higher temperature and humidity compared to the outside environment (Greenwood, 2022).

2.1.7 Genetics

The estimated genome size of the bumblebee is 274 Mb. The total sequence length may vary. The chromosome number can differ between the *Bombus* subgenera. However, most species have n = 18 (Owen et al., 1995; Wilson-Rich, 2016).

In recent findings and research, candidate genes and interesting loci are being studied, which might be linked to adaptations of bumblebees. Different functions of genes might lead to the production of muscle or detoxification proteins leading to traits as thermal tolerance or pesticide resistance, related to higher survival chances (Hart et al., 2022). Further research of genetic and epigenetic factors is essential to better understand bumblebees and their responses to stress factors.

2.2 BOMBUS PASCUORUM

This master thesis focuses on one particular bumblebee species, i.e. ***Bombus pascuorum***, also known as the common carder bee as shown in ***Figure 2.4***.

B. pascuorum is one of the most widespread bumblebees, present on different continents. They are a Western Palaearctic species. Even though they are highly abundant, they avoid cold tundra and warm steppe regions (Williams et al., 2007; Rasmont & Iserbyt, 2022). Their distribution range and habitat can vary widely, as they are found next to agricultural areas, around forests, in meadows, gardens, urban park areas, open spaces, at the side of roads, etc.

Figure 2.4: *Own photo of* B. pascuorum.

In total, 23 (Rasmont & Iserbyt, 2022) to 24 (Lecocq et al., 2015) subspecies are described. In Belgium, only three subspecies are observed: *B. p. floralis*, *B. p. freygessneri* and *B. p. moorselensis*. Lecocq et al. (2015) illustrated the distribution of the different subspecies of *B. pascuorum* in Europe as seen in ***Figure 2.5***.

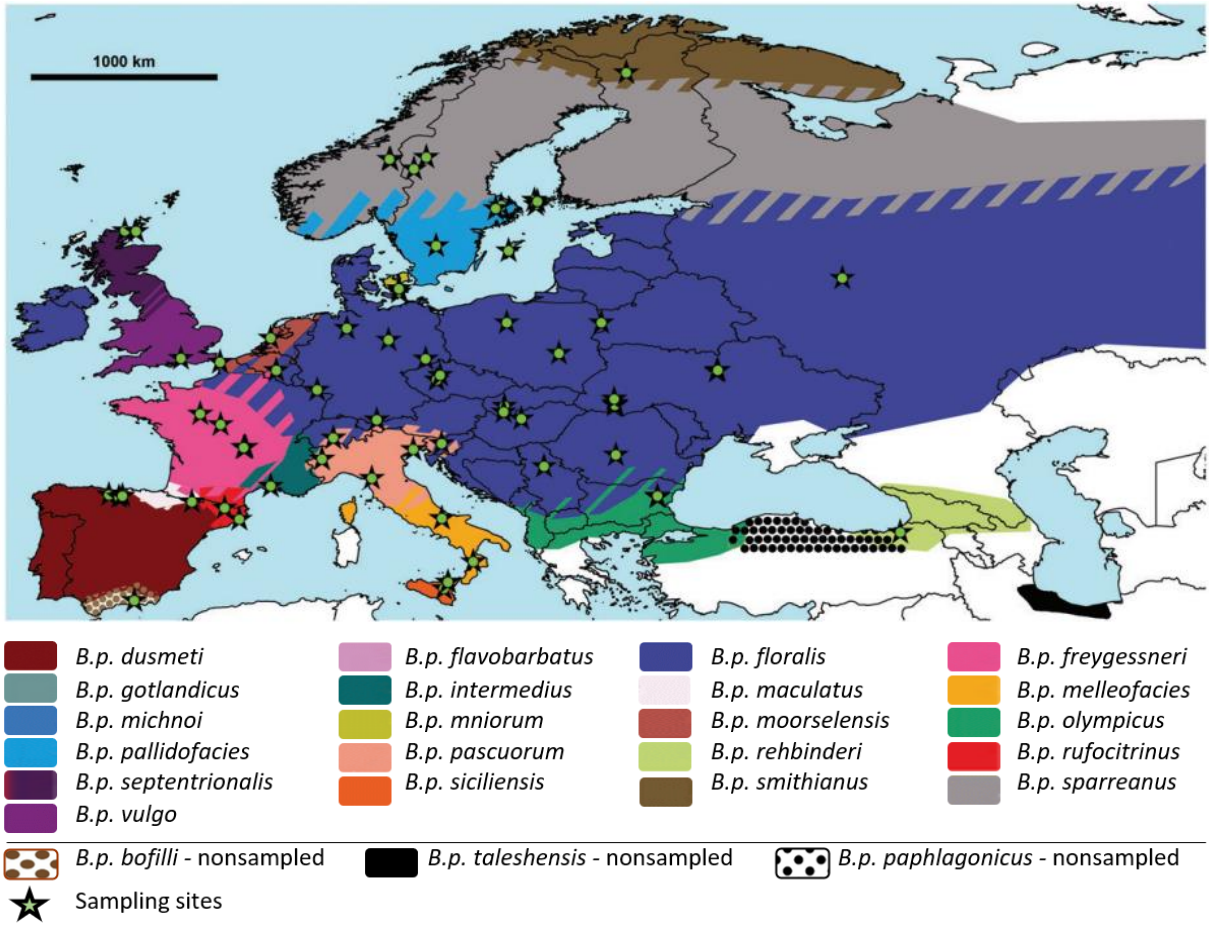

Figure 2.5*: Distribution and sampling map of* B. pascuorum *and its subspecies in Europe and surrounding regions based on traditional subspecies classification. The approximate distribution of each subspecies and sympatric areas (hatched areas) between parapatric subspecies according to the literature. The green and black stars are the sampling sites. (Lecocq et al., 2015).*

B. pascuorum belongs to the group of the ginger backs and can be recognised by its fluffy brownish back. They are social bumblebees who live in colonies which can consist of 50 up to 200 individuals. It has different phenotypical variations within its species. There are dark and light variations, where the colour on their back can vary. Both light and dark females and males exist as shown in ***Figure 2.6*** (Falk, 2015; Kos, 2019).

Figure 2.6: *Colour variations in B. pascuorum. The male dark and light variant (two left bumblebees) and female dark and light variant (two right bumblebees) (Kos, 2019).*

B. pascuorum has some specific traits worth mentioning. For bumblebees in general, queens are the biggest and drones are bigger than workers. However, for *B. pascuorum*, there is an overlap between the size of queens, workers and drones, which makes it hard to determine the sex just by size. Their average size is smaller compared to most other bumblebee species and they belong to the long-tongued bumblebees. The tongue, which is on average eight mm long, allows *B. pascuorum* to eat from flowers which are hard to reach for species with a shorter tongue (Jacquemart et al., 2019).

The flying period begins in March and ends in September/October (Falk, 2015), which makes them one of the species which can still be observed in fall. The foraging range has been studied by different researchers with different methodologies. Landscape context (rural or urban), environment and availability in nutrients play a role in the flight distances (Osborne et al., 2008). Chapman et al. (2003) estimated a foraging range of 0.5 km – 2.3 km for *B. pascuorum*. In comparison, the result for *B. terrestris* was 0.6 km – 2.8 km (Chapman et al., 2003). Darvill et al. (2004) estimated a foraging range under 312 m for *B. pascuorum* and above 312 m for *B. terrestris*. Knight et al. (2005) concluded a foraging range of 449 m and 758 m for *B. pascuorum* and *B. terrestris* respectively.

The daily rhythm of *B. pascuorum* shows an active foraging time between six a.m. and ten p.m., with each day an increase in activity during morning, reaching a maximum around midday and a decrease in the evening (Stelzer & Chittka, 2010).

2.3 STRESS FACTORS

In the last decades, especially since the 1950's, a global decline in bumblebees has been observed (Arbetman et al., 2017; Rollin et al., 2020), which is attributed to various stress factors. The European Red List of bees states that from the 68 bumblebee species in Europe, 45% shows a declining population trend and 24% are listed as threatened with extinction (Nieto et al., 2014; Votavová et al., 2022).

The Belgian Red List specifies that 20% of bumblebee species in Belgium is regionally extinct, 20% is endangered with extinction, 13.3% is endangered, 13.3% is almost endangered, 13.3% is vulnerable and only 20% is non-endangered (Drossart et al., 2019). *B. pascuorum*, subject matter of this thesis, is considered as a non-endangered species in both the above-mentioned European and Belgian Red Lists.

Bumblebees can be threatened in their survival by changes in their environment and climate, either by natural or anthropogenic factors. The main drivers of bumblebee decline are climate change, the increased use of pesticides, the occurrence of pathogens, diseases and pests, disruptions in the floral resources or loss of suitable nesting habitats, as well as reduction of genetic diversity, as shown in ***Figure 2.7***.

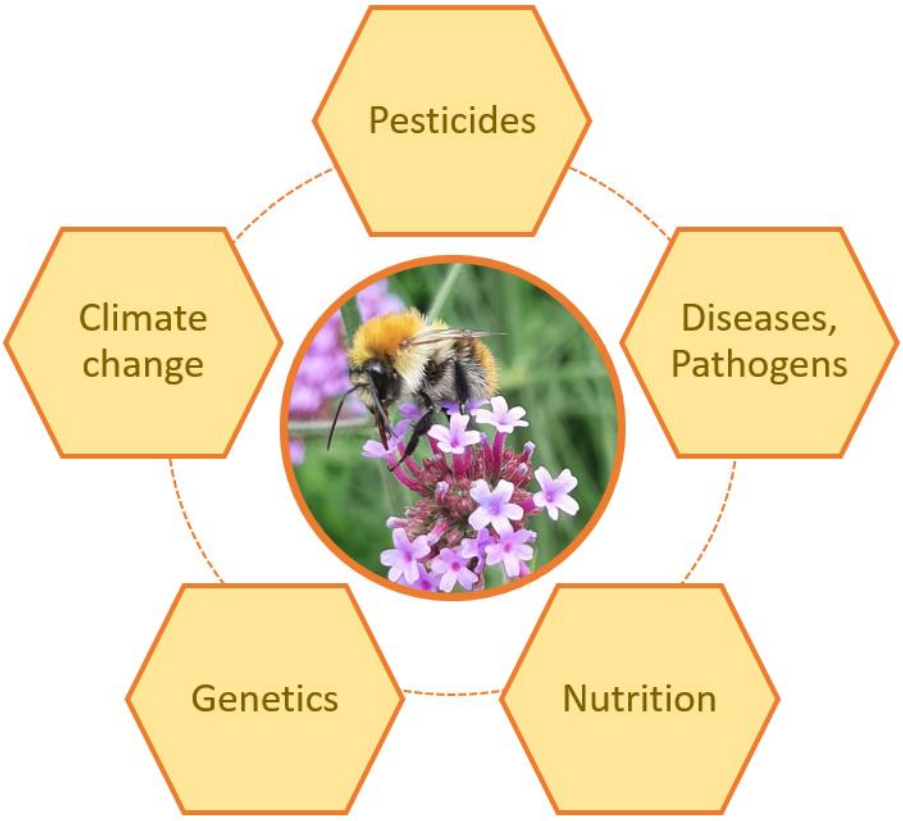

***Figure 2.7**: Own photo of* B. pascuorum *and various stress factors linked to bumblebee decline.*

All these stress factors can have a major effect on the individual bumblebee (including at genome and cell level), the colony as a whole and to a wider extent the whole ecosystem. Thus, rather than isolating one responsible stress factor, it is clear that the interrelationship of threats and combination of circumstances leads to an overall effect, resulting in a lower resilience of the bumblebee (Goulson et al., 2015; Folschweiller et al., 2020).

Bumblebees can adapt to stress factors and develop different traits e.g. morphological or behavioural, to have a higher survival chance in a specific environment. This is also called phenotypic plasticity, where individuals can express different phenotypes in response to various environmental conditions (Pigliucci et al., 2006).

Environmental factors that create stress such as rising temperatures, UV-exposure, extreme weather conditions (heat and cold waves), precipitation patterns and humidity, but also the effects of human interference, such as burning fossil fuels, fall under the factors related to climate change.

Agricultural intensification with land fragmentation and changing land-use, the increasing use of pesticides, insecticides and fertilizers, the import of non-native species and invasive plants and the introduction of new diseases, resulted in the diminishing presence and distribution of floral sources, being the main food source of bumblebees (Kreyer et al., 2004; Cressey, 2015; Maebe et al., 2021a; Iwasaki & Hogendoorn, 2022).

Within bumblebee populations, the presence and spread of endemic and introduced pathogens, diseases and viruses can cause a decline in both species' abundance and diversity. Even the presence of a locally high species' diversity could cause negative effects due to increased competition between these bee species (Åkesson et al., 2021; Iwasaki & Hogendoorn, 2022).

The survival of bees is not only influenced by environmental and anthropogenic factors, but also by some genetic factors and differences between the species. Following the phrase "survival of the fittest", the species which is best adapted to its environment and able to overcome certain threats, will be able to survive and thrive. *B. pascuorum* is considered one of the more stable bumblebee species. They are widespread and have managed to survive without sharply decreasing numbers over the years. They have a high genetic diversity, which results in a higher fitness and ability to adapt and survive different circumstances or negative stress factors in the environment (Maebe et al., 2015). In contrast, declining species have lower genetic diversity and lower fitness, with less ability to react to changes in the environment and less chances to survive. Moreover, they have a higher chance of inbreeding and are more vulnerable to diseases (Maebe et al., 2015).

As stated before, the sex of the bumblebees is determined by a single sex locus. Females are heterozygous and possess two different alleles (D,E - diploid). Males are normally hemizygous and only possess one allele (E - haploid). However, due to inbreeding or when the genetic diversity is very low, multiple copies of the same allele can lead to the production of diploid males. These are homozygous and have two identical alleles (E,E - diploid). This has serious consequences, as these diploid males do not contribute to the colony and when mating with queens this results in sterile (triploid) or unviable offspring and the colony ends up in the "diploid male extinction vortex" (Gerard et al., 2015; EIS, 2021; Pietro et al., 2022).

The reduction of genetic diversity was assumed to be linked to the 1950's loss of habitats and loss of food sources. However, research on the genetic diversity, based on heterozygosity and allele richness, in both historical and recent populations of several bumblebee species, indicated that the difference in genetic diversity between stable and declining bumblebees might already have been present in older specimens, as the historical populations of the declining species had a lower diversity compared to the stable species. Over time, a temporal stability of genetic diversity was observed for both the more stable and declining bumblebee species (Maebe et al., 2015).

Moreover, previous research has shown population structuring for *B. pascuorum* (a stable species) on a wider scale between countries in Europe (e.g. Estonia and Belgium). However this was not seen on a local scale (e.g. only Belgium). In declining species, no population structuring was detected (EIS, 2021).

Genes may be associated with the capacity to adapt to climate, temperature, altitude and also agricultural *versus* natural areas, where the changes and specific adaptations may have a positive impact on survival (Hart et al., 2022). Some mutations can affect gene transcription and change gene function (interact or activate), which results in new traits of bumblebees.

This thesis will further focus on *B. pascuorum* from agricultural and natural regions, studying various stressors: pesticides, heat stress and malnutrition. Under laboratory conditions, pesticide, thermal tolerance and nutrition experiments are performed, while also analysing morphology and the presence of pathogens, to evaluate if there are noteworthy differences which could be related to an improved ability to survive in agricultural or more natural areas.

2.4 PESTICIDES

2.4.1 Pesticides in general

Increasing the welfare of the human population, providing a safe, healthy environment in a sustainable way, is a difficult, but essential goal that people try to reach by improving technology, increasing production and learning about the ecosystem and interactions with living organisms. However, high quality and high quantity crop production is not possible everywhere, resulting in around 9% of the world population still being undernourished (Fioni et al., 2023).

Pests, diseases, insects and contamination all have the potential to destroy plants, spread plagues or decrease yields enormously, which has a negative impact on the food market, economy and welfare. Therefore, a wide range of pesticides is used in agriculture, urban areas and roadsides to protect crops, flowers and plants. The correct use of pesticides and good management of crops is essential for a safe and optimal result.

Pesticides is a general term for a range of products, which include herbicides, insecticides, fungicides, repellents, avicides and rodenticides, each with a specific target and active component (EFSA, 2023). Weeds, insects and nematodes that attack plants are well known problems, but also birds, rodents and fish can be targeted. The goal is to overcome these pests, without affecting other plants, animals and other organisms. Possible negative effects can be lower fertility, sickness, changes in behaviour, decline and death of non-target species or a negative ecological impact by contaminating water sources and soil.

In the European Union (EU), a long procedure must be followed before pesticides are approved and brought on the market. Interactions between member states, the European Food Safety Authority (EFSA) and the European Commission are essential and the whole procedure can take up to three years before a product is approved or not. In the United States, the Environmental Protection Agency (EPA) will assess the risks and evaluate the product. The US has less strict regulations than the EU, which makes it sometimes easier to bring a product on the market, leading to more products approved and used (EPA, 2023).

Pesticides can be classified based on toxicity, use or purpose, chemical composition, mode of action and source of origin. Some are biological/natural, others chemical/synthetic (Hassaan & Nemr, 2020).

Another type of classification is linked to safety, based on LD50 (50% of individuals die at this dose), where acute and chronic effects and mode of administration are important to study during research and (pre) clinical phases (Akashe et al., 2018).

Neonicotinoid insecticides (e.g. thiamethoxam) are known for their lethal and sublethal effects on bumblebees. The use of these products is forbidden in the EU (Mobley & Gegear, 2018; Baron et al., 2017). Spinosad however, an important and relatively well studied pesticide, is approved for use in Europe and will be used in the pesticide experiments referred to in this thesis.

2.4.2 Spinosad

Spinosad is a microbial bioinsecticide first reported by Mertz and Yao in 1990. It is a natural product approved for use in organic agriculture by numerous national and international certifications. The first approved use took place in the United States in 1997, as a reduced-risk insecticide on cotton. The European Commission gave its approval for use in 2007. Spinosad is a product of Dow Agrosciences, sold under the tradenames of Tracer, Success, SpinTor and Conserve1 (Mayes et al., 2003; Abdu-Allah et al., 2011; Bunch et al., 2014).

Spinosad is a large, complex molecule, containing compounds with a ring system (tetracyclic macrolytic structure). It consists of two metabolites that are produced by the bacterium *Saccharopolyspora spinosa*, which naturally occurs in the soil. This gram-positive, non-motile, filamentous micro-organism is an actinomycete part of the higher phylum of the Actinobacteria, also called Actinomycetota. Spinosad is a mixture of spinosyn A (major component with 50 to 95% of the mixture) and spinosyn D (only 5 to 50%). Spinosyn A and D are similar, except that one hydrogen atom in spinosyn A is replaced by a methyl group in spinosyn D. The chemical structure can be seen in ***Figure 2.8***. Structural changes and modifications exist, creating a variety of spinosyns with a large diversity in use (Kirst, 2010; Abdu-Allah et al., 2011).

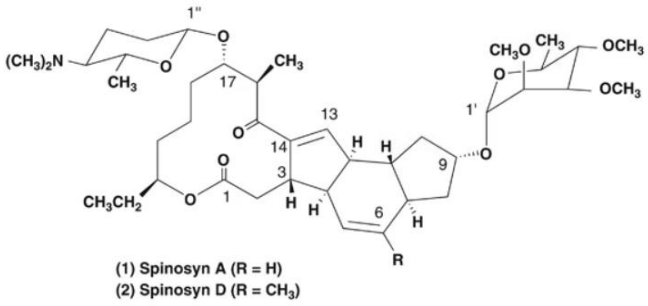

Figure 2.8: *Structure of Spinosyn A and D (Kirst, 2010).*

Spinosyns are broad-spectrum insecticides, active against Diptera, Lepidoptera, Hymenoptera, Siphonaptera and Thysanoptera (Mayes et al., 2003; Kirst, 2010; Abdu-Allah et al., 2011). Spinosad can be used on a wide variety of fruits, vegetables and grasses, where the product will kill insects including fire ants, fruit flies, mites, mosquitoes, spider mites and thrips (Bunch et al., 2014). Spinosad can be bought in spray form, where both ready-to-use and concentrated liquids are commercially available. Dust and granulated forms can be bought as well. Another application is the use of Spinosad in veterinary medicine, known as Comfortis, to kill parasites, lice and fleas on dogs and cats (Robertson-Plouch et al., 2008). When insects ingest or touch wet Spinosad, the substance will be taken up by the body and act as a neurotoxin, which targets binding sites on nicotinic acetylcholine receptors in the nervous system, causing disruption of acetylcholine neurotransmission. Spinosad is also known as a gamma-amino-butyric acid (GABA) neurotransmitter agonist and kills insects by hyperexcitation of the insect's nervous system causing spasms, paralysis and death (Kirst, 2010; Dalefield, 2017).

Spinosad is rapidly broken down by sunlight, while not easily degraded by and hard to solubilise in water. It has high efficacy, low mammalian toxicity and a good environmental profile. It is stated that Spinosad is safe for humans and pets such as cats, dogs and rabbits (Bunch et al., 2014). However, some side effects such as dry skin, hair loss, eye redness and irritation may occur and several studies have proven the danger to non-target organisms, including bumblebees. Spinosad might have lethal effects on bumblebees (shortening their survival period) or various sublethal effects (lower fertility and drone mass), which could put the (future) colony at risk. Research from Abdu-Allah et al. (2011) tested various concentrations and different ways of administration, concluding that wet contact with and oral intake of a high concentration of Spinosad is very harmful and lethal to bumblebees, while dry contact seemed to have less or no effect. As the effect of Spinosad has not been tested on all species and since there are genetic and morphological differences between the different species, future research testing the effect of Spinosad on bumblebees is essential (Abdu-Allah et al., 2011).

2.5 MALNUTRITION

With the growing world population, more food, living areas and essential resources are necessary to obtain and maintain a good living standard. Together with globalisation and the industrial revolution the boundaries are constantly being pushed, triggering a whole cascade of possible negative side effects.

Intensification of land use and agriculture leads to fragmentation of the available land and to habitat loss for numerous species. By replacing wild, untouched lands with fields where only one specific crop is cultivated (monoculture), not only habitats become limited, but also the food resources for bumblebees. Changes in agricultural cultivation methods, such as the changes in land use, abandonment of crop rotation and changes between fodder and cover crops, as well as the introduction of nitrogen fertilizers that cause eutrophication of the soil, have led to a major decline in wild plants and is therefore highly detrimental to bumblebees. Furthermore, the intensive farming of livestock might lead to a decline in wild plant and animal species as well (Nieto et al., 2014; Folschweiller et al., 2020).

In Europe, the strong intensification of agricultural productivity with large fields of homogenous crops is linked to the introduction of the European Common Agricultural Policy (CAP) in 1962, which aimed to support farmers and ensure a stable food supply. Only recently however, to halt the decline of pollinators in the EU, pollinator conservation measures have been specifically included in the CAP objectives (Mottershead & Underwood, 2020).

Pollinator friendly farming has to be implemented in future policies, as recent research has shown that the decrease of major bumblebee food sources has a negative impact. Calculations show that for Belgium, the number of hectares of cultivated Fabaceae, which is considered to be a major pollen source for most bumblebee species, has decreased from 163,700 ha in 1908 to less than 2,500 ha in 1985 (Folschweiller et al., 2020). Research has confirmed that there is a clear link between the decline of long-tongued bumblebees and the greatly reduced use of red clover (*Trifolium pratense*) as natural “green” manure in agriculture (Folschweiller et al., 2020).

Also the decline of the thistles from the Cardueae tribe of the Asteraceae family is regrettable. Pollen from the Asteraceae family are seldom found in queens’ and workers’ pollen loads, implying it is

considered as a non-optimal diet to feed the offspring. However, for drones, thistles play a vital role in their diet. The nectar resources of *Carduus, Centaurea* and *Cirsium* species are essential energy providers for the drones at the end of the summer when they mate (Vray et al., 2017). Therefore, the Belgian and European strict legal regulations against thistles (which are considered harmful weeds in agriculture), clearly have negative consequences on bumblebees and require a profound review.

Moreover, land-use change for recreational, industrial and mining purposes, depletion of natural resources, together with increasing pollution are important contributors to the decrease in habitat and food source diversity and quality for the pollinators.

Invasive plant species can outcompete native plants that serve as an essential food source for animals. Disappearance of native plants may lead to bumblebee decline. However, there are some exceptions, where invasive exotic plant species are frequently visited and can serve as an alternative food source for bumblebees. A prime example is *B. pascuorum* being strongly attracted to the invasive plant species, Himalayan balsam or *Impatiens glandulifera* as a new food source (Folschweiller et al., 2020).

More areas are required with a high variety and amount in food sources for bumblebees where the flowers have the opportunity to grow. Examples are: untouched natural areas with permanent plant species, grasslands, heathlands, hedges, but also gardens in an urban environment (Vanbergen, 2013). Even in forests, where flowers may not be abundant as the whole area is covered in shadow by the crowns of the trees, open areas and the edges of the forest may be abundant with flowers and thus more suitable for bumblebees.

2.6 HEAT STRESS

2.6.1 Climate change

World evolution has been accompanied by shifts in climate, alternating between extremely cold (ice ages) and warmer periods. During the last century however, global temperature rises not only due to natural but also anthropogenic factors, resulting in extreme temperature shifts accompanied by storms, floods, droughts and forest fires which wipe out complete eco-systems.

Climate change has become one of the most important topics in the past decades as it has a huge impact on each life form and all ecosystems on earth. The endurance of ecosystems, as well as the survival of numerous plant and animal species and consequently human existence, may depend on our goodwill and capability to reduce global warming and confine climate change in the years and decades to come. In order to keep the upward trend under 1.5°C, as was agreed by the Paris Climate Accords in 2015, greenhouse gas emissions must be reduced by about 50% by 2030 in order to reach a net zero level by 2050 (UNFCCC, n.d.).

Since bumblebees live in various climates all around the world, at different temperatures and altitudes, also they will feel or suffer the effects of climate change. For example, floral resources and food availability may be affected. Warm temperatures and changed weather conditions have a direct effect on bumblebees' geographical native habitat range. Research by Kerr et al. (2015) confirmed that while they want to escape extreme hot temperatures in southern geographic areas, they do not always shift to the cooler north. The difficulty to relocate to other geographic areas squeezes them into ever smaller suitable habitats.

2.6.2 Temperature limits

Rising temperatures and thermal stress may affect bumblebees' behaviour and morphology and can lead to new gene adaptations (Maebe et al., 2015; Oyen & Dillon, 2018; Kenna et al., 2021; Maebe et al., 2021a; Maebe et al., 2021b; Hart et al., 2022).

Behavioural changes include mass migration, burrowing and changes in emerging and foraging rhythms. Related to **morphology**, it is known that body size, wing size, hairiness and colour patterns have an impact on how well they can regulate their heat (Maebe et al., 2021b). As to body size, Bergmann's Rule is applicable to bumblebee species. This rule states that large individuals will be more suited to live in colder environments as they have a lower surface/volume ratio and will lose less heat, whereas small individuals will have more problems to retain heat as they have a larger surface/volume ratio. As fur provides insulation, it was observed that bumblebees with longer hairs tolerate colder temperatures better than bumblebees with short hairs. As heat loss can occur at wing extremities, the bumblebees with shorter wings relative to their body size were more cold resistant. This is in line with Allan's rule, where protruding body parts should be shorter in colder regions and longer in warmer areas (Peat et al., 2005). Colour patterns are linked to reflectance and absorption of heat. In different animal species, black is known to be important for absorption of solar radiation. In bumblebees, the black colour is seen around the central thorax, where heat is generated during flight, which might help to reduce flight cost/energy. (Maebe et al., 2021b). More dark colour could be linked to colder environments. However, Peeters et al. (2012) mentions that dark species are mostly present in tropical regions.

Apart from the above-mentioned morphological adaptations, some other traits are essential for bumblebees to survive cold/hot climates. Bumblebees are cold adapted insects. They are described as heterotherm as they can regulate their heat across a broad range of temperatures, from using only environmental temperatures as a heat source, to producing their own heat. Their ability to perform the latter is also called facultative endothermy (Dzialowski et al., 2014; Oyen & Dillon, 2018).

2.6.2.1 Cold resistance

Bumblebees can regulate their own temperature (thermoregulation). It is crucial that bumblebees are able to generate their own warmth, because they cannot fly if their own temperature is below 37°C. Bumblebees can vibrate their wings and actively use flight muscles to create heat, resulting in a higher thorax temperature. Another internal way to generate heat is called substrate cycling, where enzymes fructose diphosphatase and phosphofructokinase work to burn adenosine triphosphate (ATP) (Maebe et al., 2021b).

Bumblebees are freeze-avoidant and prevent freezing through physiological and biochemical means (e.g. using polyols, sugars or anti-freeze proteins), lowering the supercooling point to avoid formation of ice crystals (Bale, 1996). Bumblebee queens can hibernate and can regulate their metabolism, activity levels and energy reserves to survive the winter.

In decreasing cold temperatures, bumblebees can go in total paralysis seen as a chill coma. In general, the neuromuscular functions are lost first. The bumblebees fall over and become unresponsive. Important is that this state is reversible. When temperature increases, they will become more active again. However, if the temperatures keeps decreasing, bumblebees will die (Oyen and Dillon, 2018).

2.6.2.2 Heat tolerance

The heat shock protein (hsp) genes and heat shock response cascade are mechanisms to handle heat stress (Maebe et al., 2021b). Another way to lose heat and prevent overheating is by increasing the flow of warm haemolymph and transporting this from the thorax to the abdomen (Heinrich, 1976).

Ongoing tests and future research might use genomic approaches e.g. Restriction-site-Associated DNA sequencing (RADseq) and transcriptomics (RNAseq), to identify genes linked to interesting above-mentioned traits (e.g. muscle function, heat shock proteins, cell-membrane-related proteins), adaptations and functions important to regulate temperature (Kenna et al., 2021).

2.6.2.3 CT_{min} and CT_{max}

Temperature tolerance limits vary between different bumblebee species. When the temperature drops, bumblebees can go in a "hibernation" state which allows them to survive this period with unsuitable thermal conditions. The same is possible for warm temperatures where they go in an "aestivation" state (Maebe et al., 2021b).

The critical temperatures, CT_{min} and CT_{max} are minimal and maximal temperature extremes, which correspond to the coldest and hottest values respectively, at which bumblebees are still able to control their muscles and have the ability to fly and move.

Research from Maebe et al. (2021a) focussed on the thermal tolerance limits of three *B. terrestris* subspecies. Using a temperature cycle in a specialised incubator, the experiments resulted in a CT_{min} of around -4.5°C and a CT_{max} of around 50°C.

Research from Oyen and Dillon (2018) concluded that the *B. impatiens* species had a CT_{min} of approximately 4°C, ranging from 1.4°C to 8°C and a CT_{max} of approximately 53°C, ranging from 42°C to 65°C. Moreover, they stated that age and feeding status have only little effect on the tolerance limits.

In literature, exact critical temperatures of *B. pascuorum* species have not been mentioned yet. As the response to temperature is rather species-specific, further research is essential, focussing on all the different species.

2.7 PATHOGENS

Diseases caused by pathogens, parasites and viruses can have a negative impact on bumblebees. Hence, the increased spread of pathogens is one of the key factors linked to bumblebee decline.

Infections with pathogens can occur naturally, but also because of the spill-over effect, where the pathogen is transmitted from commercial bumblebees to wild bumblebees. The reverse, i.e. the spill back effect, where wild bumblebees infect commercial ones, is also possible, however less documented (Martin et al., 2021). Moreover, pathogens not only spread between bumblebee species, but also between honeybees and other pollinators, e.g. the deformed wing virus (DWV) which spreads from honeybees to bumblebees (Fürst et al., 2014; Alger et al., 2019).

In nature, vertical and horizontal gene transfer are frequently used transmission pathways. In **vertical transmission,** genetic information (e.g. a virus or a plasmid containing antibiotic resistance genes) is passed on to the next generation. The transfer happens from "parent to offspring" in bumblebees

(Chen et al., 2006; Sanseverino et al., 2018). In **horizontal transmission**, genetic information is transferred to unrelated individuals. For bumblebees, horizontal transmission happens between individuals of the same generation, either via a direct or indirect route. The direct route can occur via air- or foodborne infection or via sexual intercourse (venereal infection). The indirect route requires an intermediate host, who transmits the virus from one bumblebee to the other (e.g. mosquito) (Chen et al., 2006; Piot, 2020; Votavová et al., 2022).

Bumblebee pathogens and viruses are transmitted via the above-mentioned pathways. Pathogens can spread horizontally, within a colony or between colonies in case the territories and foraging areas overlap, via contact with flowers, pollen, nectar and faeces. A vertical spread of pathogens might occur when males mate with their future queens. The transmission mechanism and range of effects depend on the pathogen/virus. In some cases, the effects are limited, in others more severe and the results may vary between natural *versus* laboratory circumstances. Some infections with pathogens not only affect the survival rate, but have other consequences as well e.g. on reproduction (Chen et al., 2006; Piot, 2020; Votavová et al., 2022).

The most prevalent viruses are the Deformed wing virus (DWV), Acute bee paralysis virus (ABPV), Black queen cell virus (BQCV), Sacbrood bee virus (SBV), Lake Sinai virus (LSV) and Loch Morlich virus (Ocepek et al., 2021; Pascall et al., 2021). Well known disease causing parasites in bumblebees are ***Nosema*** (a microsporidian) and ***Crithidia*** and ***Apicystis*** (two protozoan parasites) (Ocepek et al., 2021). Prevalence of these pathogens in bumblebees can be detected using PCR diagnostic methods and molecular primers. In general, the incidence can be estimated at around 20% for both *Nosema* (total of *N. bombi* and *N. ceranae)* and *Crithidia bombi,* while at 50% for *Apicystis bombi*. Half of the bumblebees were infected with one parasite, whereas around 10% had multiple parasites (Vanderplanck et al., 2019; Ocepek et al., 2021). Another parasite is the **conopid fly**, which can visually be detected in the abdomen during a dissection or by using genetic barcoding.

2.7.1 *Nosema*

Nosema (class Microsporidian) is a parasite which can affect bees. The name was recently reclassified to "*Vairimorpha*" for the parasites specifically infecting bumblebees. *Nosema* parasites will infect the gut and more specifically the midgut of the host. Spores are ingested, reach the midgut, germinate, infect the other cells in the digestive tract and are finally excreted (Piot, 2020). The faeces then contaminate the food sources of others. The spores can be dormant (inactive and no multiplication) for a long time before causing mortality or making bees more susceptible to other diseases. Infection has negative effects on reproduction. Infected queens produce smaller colonies and offspring with lower fertility (Grupe & Quandt, 2020). *N. bombi* (*V. bombi*) and *N. ceranae* (*V. ceranae*) are two main parasites. *N. ceranae* mainly occurs in Asiatic honeybees (*Apis cerena)*, but is now also infecting other honeybees and bumblebees. *N. ceranae* is one of the potential factors causing "Colony Collapse Disorder" (disappearance of colonies) (Kim et al., 2017). *N. bombi* was linked to a reduced life span of workers and colony fitness. The prevalence of this species varies, depending on the gender of the bumblebee, where males are more affected than females (Piot, 2020).

2.7.2 Crithidia

Crithidia bombi is the most prevalent species of the genus *Crithidia,* a trypanosome infecting the gut of honey- and bumblebees. Infection happens via an oral-faecal transmission route. In beneficial environments, it is a commensal and considered as benign, not causing any serious risks. However, in environments with reduced nutrition, the virulence increases, leading to reduced fitness of bumblebees and a 50% higher mortality. The bees lose the ability to distinguish flower colours and visit the wrong flowers containing less nectar which will lead to starvation (Gegear et al., 2006).

2.7.3 Apicystis

From the genus *Apicystis*, the species *A. bombi* is a neogregarine pathogen causing increased mortality, reduced fat body and increased sensitivity to sucrose in workers and queens. Infection happens via an oral-faecal transmission route. Infected bumblebee queens are unlikely to survive hibernation or establish a new colony (Maharramov et al., 2013; Piot, 2020).

2.7.4 Conopidae

The family of the Conopidae (conopid flies) including the genus *Conops*, are also called thick-headed flies and are parasites which infect and parasitise bees and wasps. An adult female parasite fly injects one egg inside the foraging bumblebee's abdomen. This egg hatches and becomes a larvae, which feeds on the bumblebee's haemolymph and gut, resulting in the inevitable death of the host after ten to twelve days. The larvae pupates, forming a red cocoon inside the abdomen, overwinters inside the host and will emerge as an adult fly the following spring (Malfi et al., 2018). ***Figure 2.9*** shows the development of a conopid fly.

The few studies which focussed on conopid flies infecting bumblebees, revealed that it is beneficial for flies to develop in larger bumblebees. Moreover, a change in the bumblebee's behaviour is seen as the parasite manipulates the host to display grave digging activities to increase its own fitness. In the future, more specific bumblebee species and interactions on behaviour could be studied (Malfi et al., 2014).

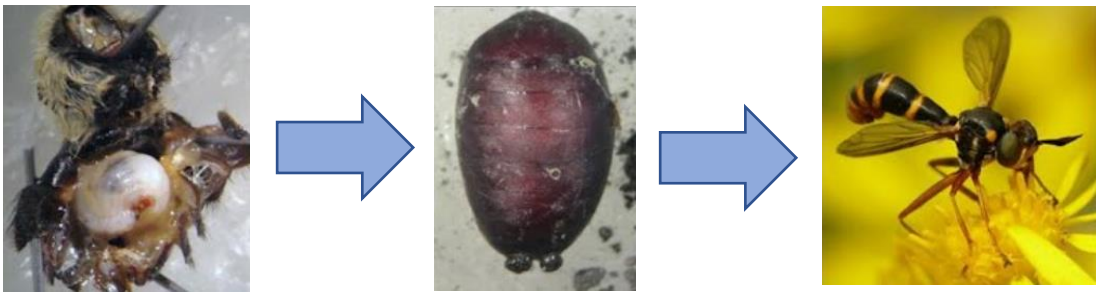

Figure 2.9: *Development of a conopid Fly. Larvae inside the abdomen of a bumblebee (left), pupa of a conopid fly extracted from the bumblebee's abdomen (middle), adult conopid fly* (Conops spp.) *(right). The adult fly will lay eggs in bumblebees and the life cycle will start again. (Malfi, n.d.).*

2.8 HYPOTHESES

The aim of this thesis is to investigate if there are noticeable visual, behavioural and morphological differences between bumblebees from agricultural habitats and bumblebees originating from more natural habitats in Belgium. This thesis does not include differences at genome level, as more research and knowledge is required to study specific candidate genes and functions linked to these genes.

Several experiments (pesticide exposure, malnutrition and thermal tolerance) were performed to determine whether bumblebees from said habitats respond differently to stress factors or have developed specific traits/adaptations that can be linked to their specific habitat characteristics.

The first hypothesis assumes that bumblebees living in agricultural areas, will survive longer with a high pesticide concentration compared to those in natural areas, since the former are more likely to have been exposed to pesticides before and have built up a tolerance.

The second hypothesis assumes that bumblebees living in areas with limited food resources are able to survive longer with low food concentrations compared to bumblebees that are used to high food availability. Agricultural areas with fields of homogenous crops and limited presence of flowers are assumed to have lower food resources, whereas natural areas can have diverse flowering populations over a long period of time without interruptions.

The third hypothesis assumes that there is no clear difference in the effect of extreme temperatures between bumblebees from the different agricultural and natural areas tested, due to the small geographical scale (Belgium). When comparing *B. pascuorum* from more distinct and remote areas (different countries or continents), one might expect that some traits are related to climate differences. Likewise, their survival capacity at certain temperature extremes may differ.

The evaluation of the experiments took into account the presence of pathogens, possible morphological differences and other factors, such as location specific effects that might influence the results. For example: temperature regulation differs for small and large bumblebees or if viral pathogens were present in certain individuals, their immune system would be weaker and they might not survive due to their illness or reduced ability to cope with a stress factor, not because of the experimental settings alone.

3. MATERIALS AND METHODS

3.1 EXPERIMENTAL SET-UP

3.1.1 Agricultural versus natural habitats

A total of 738 bumblebees were collected on three different occasions in August and September 2022 from six different locations in Flanders (Belgium), including three more natural and three agricultural locations. The locations were selected based on satellite data (google maps), choosing areas with suitable characteristics within a radius of one km. ***Figure 3.1*** shows all locations on a map. In this thesis, we refer to the bumblebees caught at natural and agricultural locations as "N" (Dutch: natuurlijk) and "L" (Dutch: landbouw) respectively. ***Appendix 1*** gives more detailed information about the six locations, with exact coordinates, dates when the bumblebees were caught and flowers present.

For the more natural locations, a selection was made from areas in and around forests, grass- and heathlands, where human management of plants and trees is limited. These areas are assumed to contain less or even no pesticides which are normally used on crop fields and to have a higher flower diversity and more food.

The agricultural locations were situated close to cultivated fields with crops, assuming the use of yield-inducing and crop protecting compounds. In these areas, a higher concentration of pesticides might be present and due to fragmentation and lower diversity, less flowers will be present in time, compared to the natural places.

Only *B. pascuorum* bumblebees were caught and this on days with favourable weather conditions: mostly sunny, no or limited rain, no strong wind and moderate to high temperatures (>17°C and <30°C). The *B. pascuorum* specimens which were caught, visited different plants such as red clover (*Trifolium pratense*), common hemp-nettle (*Galeopsis tetrahit*), Himalayan balsam (*Impatiens glandulifera*), field thistle (*Cirsium arvense*), common comfrey (*Symphytum officinale*) and common heather (*Calluna vulgaris*).

After capture, the specimens were put into small reusable housing boxes containing 15 to 20 bumblebees and transported to Ghent (Faculty of Bioscience Engineering, Campus Coupure), where they were given at least 24 hours to adapt while being placed in a basement under controlled and standardized bumblebee rearing conditions (25°C room temperature and 60%-65% humidity) and receiving 50% sugar water *ad libitum*. After being used in one of the different experiments, bumblebees were stored in a freezer (-20°C) in individual, labelled 1.5 mL Eppendorf tubes.

3.1.2 Data

Appendix 2 contains different lists with data from all 738 bumblebees from each location, including the batch number, label, gender, the experiment performed and corresponding results. Experiment data was collected in separate datasets for statistical evaluation and converted from Excel to csv files for use in R Studio (version 4.1.2), these excel files can be found in ***Appendix 2*** as well.

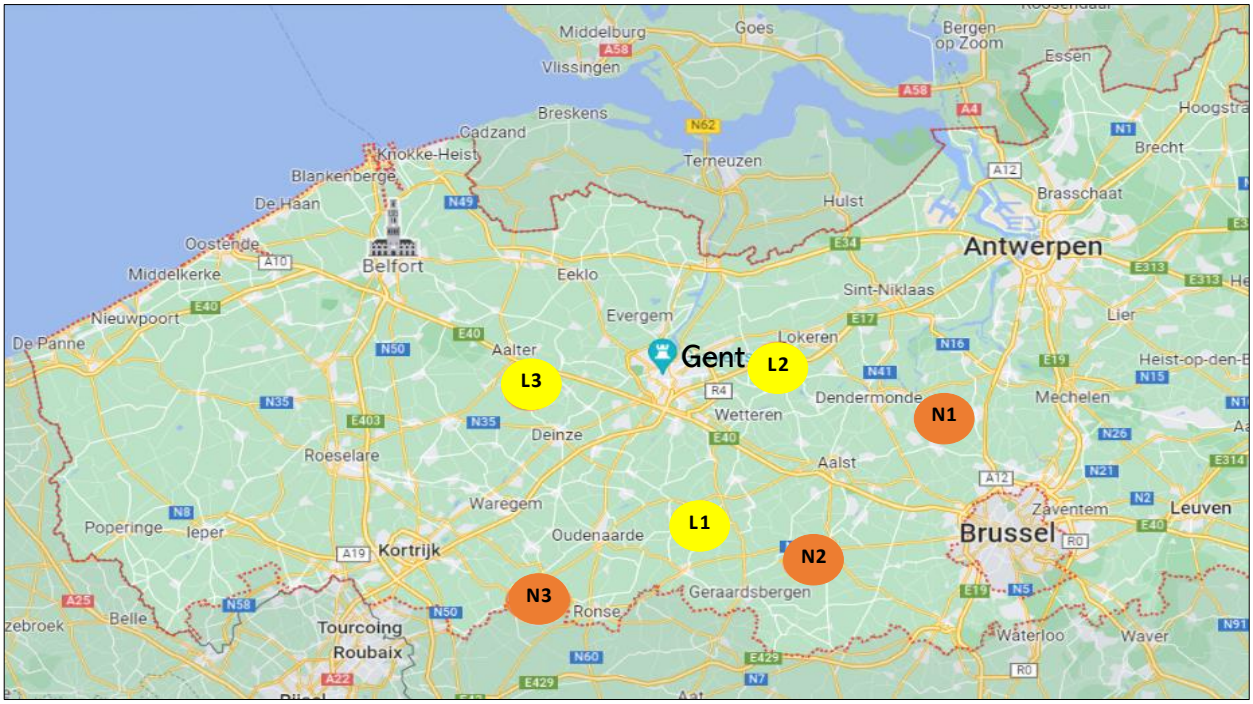

Figure 3.1: *Agricultural (Yellow: L1, L2 and L3) and Natural (Orange: N1, N2 and N3) locations where bumblebees were caught on a map of Flanders (Belgium). L1 = Zwalm, L2 = Beervelde, L3 = Poeke, N1 = Buggenhout, N2 = Ninove, N3 = Kluisbergen.*

3.2 BUMBLEBEE EXPERIMENTS

Bumblebees were placed into individual plastic tubes, connected to a syringe filled with three mL of sugar water solution as shown in ***Figure 3.2***. The bumblebees were all housed in the same room with the same standardized temperature (25°C) and humidity conditions (60-65%) to minimise variation.

Figure 3.2: *Individual housing of bumblebees in reusable tubes, connected to syringes with sugar water.*

Materials to make sugar water:

- Sugar
- Water (<u>not</u> demineralised)
- Scales
- Stove

- Spoon
- Measuring cylinder
- Erlenmeyer
- Pot

To make a "normal" 50% sugar water concentration, one kg of sugar was measured on a scale and added to 2L of warm water. The mixture is stirred until all sugar is dissolved and left to cool down before being given to the bumblebees. To make a 25% sugar water concentration, 500g sugar was added to 2L water.

3.2.1 Pesticide experiment

Previous research by Abdu-Allah et al. (2011), performed on *B. terrestris*, states that a Spinosad concentration of 400 µg/mL is lethal for bumblebees, whereas a lower concentration of 0.4 µg/mL is harmless, having no lethal or sublethal effect and showing no differences compared to the controls. With this knowledge, two intermediate Spinosad concentrations were used: 40 µg/mL and 4 µg/mL for the first experiment. For the second experiment concentrations of 0.4 µg/mL and 0.04 µg/mL were used.

Materials:

- Spinosad concentration (120 g/L) (Dow AgroSciences – Insecticide Spinosad – made in UK)
- Sugar water (50%)
- 20 – 200 µL Pipette (BRAND – Transferpette S – made in Germany)
- Yellow tips (Kima – yellow tip – no. 18260 – made in Piove di Sacco, Italy)
- Erlenmeyer
- Spatula
- Lab coat, goggles and gloves
- Syringes and individual housing tubes

Textbox 3.1*: Calculations for pesticide concentration.*

Concentration of pesticide needed

C = concentration [g/L] (C = m / V)

Pesticide **P2** = 40 µg/mL (= 0.04 mg/mL)

V1 = (C2*V2)/C1 = (0.04 mg/mL*200 mL)/(120 mg/mL) = 0.06667 mL = **66.667 µL**

Pesticide **P1** = 4 µg/mL (= 0.004 mg/mL)

V1 = (C2*V2)/C1 = (0.004 mg/mL*200 mL)/(120 mg/mL) = 0.006667 mL = **6.667 µL**

The initial concentration of Spinosad in the bottle was 120 g/L. Concentrations P1, P2, P3 and P4 were obtained in an Erlenmeyer by making dilutions in a safety flow cabinet, based on calculations shown in ***Textbox 3.1***. For P1 and P2, 200 mL of sugar water and the correct amount of Spinosad was added.

The solution was put in syringes, each containing three mL of the substance. To make the P3 and P4 concentrations, two mL was taken from a syringe from P1 and P2 respectively, where sugar water was added to obtain 200 mL of a x100 dilution.

The **first pesticide experiment** with concentrations P2 = 40 µg/mL, P1 = 4 µg/mL and a control group P0 = 0 µg/mL was performed on 162 bumblebees (159 females and three males). The bumblebees were randomly selected by taking individuals from all the different locations, taking into account their behaviour (mixing slow/less active with active bumblebees) and placing them in individual housing tubes. ***Table 3.1*** shows the P0, P1 and P2 groups containing 53, 54 and 55 bumblebees respectively. In total 75 agricultural and 87 natural bumblebees were tested.

***Table 3.1**: # bumblebees from each location (agriculture = L and natural = N) used in first pesticide experiment per concentration group (P0 = 0 µg/mL, P1 = 4 µg/mL and P2 = 40 µg/mL).*

LOCATION	PESTICIDE CONCENTRATION			TOTAL	
	P0 (0 µg/mL)	P1 (4 µg/mL)	P2 (40 µg/mL)		
L1	10	10	10	30	75 L
L2	5	7	6	18	
L3	9	9	9	27	
N1	10	9	11	30	87 N
N2	10	10	10	30	
N3	9	9	9	27	
TOTAL	53	54	55	162	

The **second pesticide experiment** used x100 dilutions of the concentrations used in the first experiment, i.e. P4 = 0.4 µg/mL, P3 = 0.04 µg/mL and a new control group P0 containing no Spinosad. In total, 99 female bumblebees were used, randomly divided over the three concentration groups. ***Table 3.2*** shows the P0, P3 and P4 groups containing 29, 35 and 35 bumblebees respectively. In total 51 agricultural and 48 natural bumblebees were tested.

Data was collected, listing the bumblebee's original location, start and end time of the experiment and the number of days the bumblebees were able to survive with the corresponding concentration. With this data, survival plots, also called Kaplan-Meier curves, were plotted and statistically evaluated using R Studio.

***Table 3.2**: # bumblebees from each location (agriculture = L and natural = N) used in second pesticide experiment per concentration group (P0 = 0 µg/mL, P3 = 0.04 µg/mL and P4 = 0.4 µg/mL).*

LOCATION	PESTICIDE CONCENTRATION			TOTAL	
	P0 (0 µg/mL)	P3(0.04µg/mL)	P4 (0.4 µg/mL)		
L1	5	6	6	17	51 L
L2	5	6	6	17	
L3	5	6	6	17	
N1	5	5	5	15	48 N
N2	5	7	7	19	
N3	4	5	5	14	
TOTAL	29	35	35	99	

3.2.2 Nutrition experiment

Materials:

- Sugar water (50% and 25%)
- Syringes and individual housing tubes

For the nutrition experiment, 109 bumblebees (99 females and ten males) were placed in individual housing tubes. All received three mL of sugar water, containing 50% sugar for the control group S0, while the S1 group received a reduced amount of only 25% sugar.

When a syringe was empty during the experiment, bumblebees received another three mL of the same solution. The time of death was monitored and the number of mL consumed by each individual was noted.

When collecting the third batch at the end of September, no bumblebees were found at location L1 and N1. Therefore, the nutrition experiment was performed with bumblebees from locations L2, L3, N2 and N3 only. The available bumblebees were divided into balanced groups as shown in ***Table 3.3***. Again, bumblebees were randomly selected from each location to be put in the S0 (50% sugar, control) or S1 (25% sugar) group.

Table 3.3*: # bumblebees from each location (agriculture = L and natural = N) used in nutrition experiment (S0 = 50% and S1 = 25%).*

LOCATION	SUGAR CONCENTRATION		TOTAL
	S0 (50%)	S1 (25%)	
L2	15	14	57 L
L3	14	14	
N2	17	17	52 N
N3	9	9	
TOTAL	55	54	109

3.2.3 Temperature experiment

Materials:

- Incubator (PHCbi – MIR-254-PE incubator – made in Japan)
- Infrared camera: (Optris – PI 160 IR camera – made in Berlin, Germany)
- Normal camera: (Logitech – c920 HD Pro webcam – made in Lausanne, Switzerland)
- 15 plastic cylindrical tubes with a plastic meshed top and wooden plungers

To determine the CT_{min} and CT_{max}, bumblebees were placed in individual tubes inside an incubator which follows a temperature cycle ("COOL program") as shown in ***Figure 3.3***. First, the temperature is held stable at 25°C for 15 minutes, then the temperature gradually decreases to -10°C over a period of one hour and ten minutes. Afterwards, the temperature is increased over a period of one hour and 40 minutes until 25°C is reached again. The temperature is held stable over a period of 30 minutes. Finally, the temperature is increased gradually up to 60°C over a period of one hour and 40 minutes. A complete cycle takes five hours and 15 minutes.

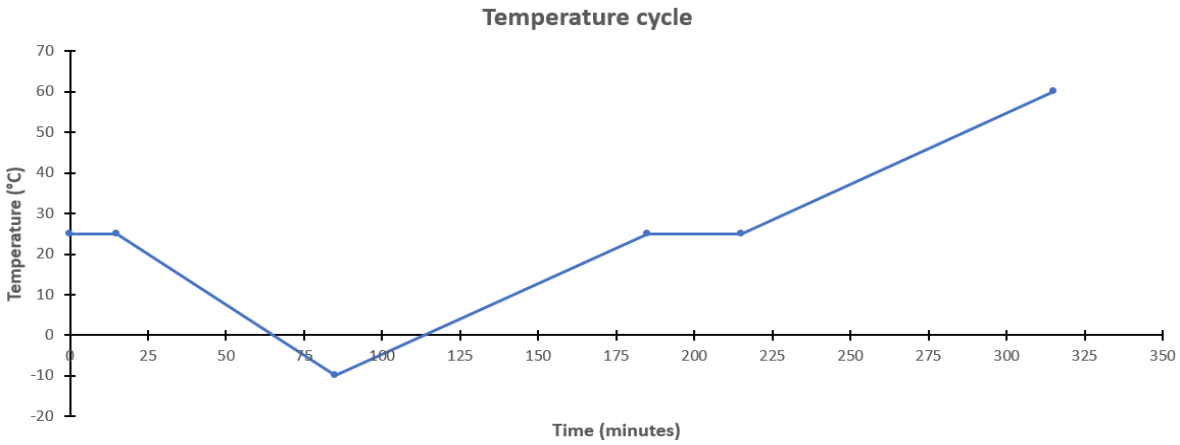

Figure 3.3: *Temperature experiment: temperature cycle followed in the incubator (y-axis = temperature; x-axis = time).*

The experiment data was collected by filming the bumblebees with a normal and an infrared camera as seen in ***Figure 3.4***, using Logitech Webcam and PIX Connect Software respectively. The footage from the normal camera was used to determine the time at which each bumblebee "falls" (the point where they lose muscle control). Correspondingly, the CT_{min} and CT_{max} were determined at the falling point via the infrared footage with PIX connect. The temperature of the surrounding area (min, max and mean) was measured.

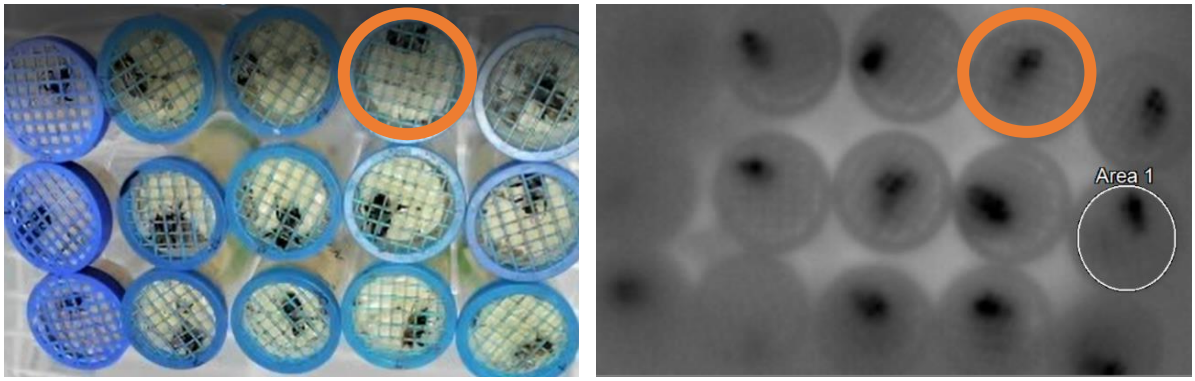

Figure 3.4: *Snapshot from webcam (left) and infrared (right) recordings. The orange circle shows the same location in both videos. "Area" is used to determine the environmental temperature at that position.*

Ten temperature experiments were performed with 15 bumblebees each (a total of 150 bumblebees). For each test, bumblebees were taken from different locations, mixing males and females and placed at random positions in the incubator. Data was gathered about the bumblebee's original location, label, extra label corresponding with the video in which they were used and their position in the incubator, both critical time points during the cold and hot period and the corresponding temperatures.

3.2.4 Morphology evaluation

Materials:

- Transparent sheets
- Transparent tape

- Dissection set: tweezers, scalpel and small scissors
- Scales (Trendfield – scale 50g/0.001g – made in China)

The morphology of all 738 bumblebees was studied. For each bumblebee the mass, the length of the radial cell of the right and left forewing and the length of all leg compartments of the left middle leg were measured. To determine if there is a significant difference between bumblebees from the different locations (including between males and females) the averages for the above-mentioned measurements were compared. By including the left and right forewing, it is possible to evaluate wing symmetry, focussing on 13 specific landmarks. This test was not performed due to the limited timeframe for this thesis. However, the morphology sheets remain available online making future integration and evaluation of the data possible.

Transparent paper was used to make morphology sheets of the bumblebees' forewings and left middle leg as seen in ***Figure 3.5***. After compiling all morphological sheets, photos were taken of the sheets together with a ruler. Measurements were determined with the *ImageJ* program that calculates the lengths between two points in mm, compared to the predetermined scale and pixel. The data collected allows a statistical analysis in R Studio.

Figure 3.5: *Morphology sheet detail: front wings and left middle leg of bumblebee with label "L2E5".*

3.2.5 Pathogen detection

In this thesis, we focus on three specific bumblebee pathogens "*Vairimorpha*", "*Crithidia*" and "*Apicystis*". Our protocol does not detect viruses or other parasites. Pathogen detection was performed on all 738 bumblebees. The protocol can be divided into three major steps: sample preparation, DNA extraction and qPCR (quantitative polymerase chain reaction). Note: The number of freeze/thaw steps was minimised to maintain the best possible quality of each sample.

3.2.5.1 Sample preparation

Materials:

- Dissection set (scissors, tweezers, dissection needles and plate)
- 20 – 200 µL Pipette (BRAND – Transferpette S – made in Germany)
- Yellow tips (Kima – Yellow tip – no. 18260 – made in Piove di Sacco, Italy)
- Eppendorf tubes (Eppendorf – Safe-Lock tubes 1.5 mL – no. 0030 120.086 – made in Hamburg, Germany)
- 100% ethanol (Chemlab – Ethanol abs. 100% a.r. (2.5 L) – no. CL00.0505.2500 – made in Zedelgem, Belgium)
- Deionised water
- Glass cylinder
- Nuclease free water (Promega – Nuclease free water – no. P119E – made in Madison, USA)
- Cardboard storage box

During sample preparation, bumblebees were pinned firmly to a dissection plate, where the abdomen was carefully cut open to extract its contents. A 70% ethanol solution was made by mixing 70 mL of 100% ethanol and 30 mL of deionised water in a glass cylinder. All dissection materials were wiped clean with this solution before starting and after finishing each sample. The contents of the abdomen was extracted using tweezers and placed in a new 1.5 mL Eppendorf tube. To extract as much as possible, including all left membranes and fat tissue, a tip was used to clean the inside tissue wall. The tubes were labelled and stored in a cardboard box (81 samples in one box) in the freezer at -20 °C.

For the negative controls, 60 µL nuclease free water was pipetted in a new Eppendorf tube, in which the (disinfected) tweezer and needles were held for approximately ten seconds. These 13 samples should not contain any pathogens.

3.2.5.2 DNA extraction

Materials:

- InstaGene Matrix (BIO RAD – 20 mL – no. 7326030 – made in USA)
- Proteinase K (Thermofisher scientific – 5 x 1 mL – no. EO0492 – made in Lithuania)
- 2 – 20 µL, 20 – 200 µL, 100 – 1000 µL Pipettes (BRAND – Transferpette S – made in Germany)
- Tips
    - Blue tips (Kima – Blue tip – no. 18172 – made in Piove di Sacco, Italy)
    - Yellow tips (Kima – Yellow tip – no. 18260 – made in Piove di Sacco, Italy)
- Eppendorf tubes (Eppendorf – Safe-Lock tubes 1.5 mL – no. 0030 120.086 – made in Hamburg, Germany)
- Automatic stirrer (Prolabo – Rotamag C14 – made in France)
- Vortex (L.E.D. Technology – Heidolph REAX 2000 – no. 54119 – made in Germany)
- Mini centrifuge (Avantor – VWR Galaxy Ministar microcentrifuge – made in Korea)
- Incubator
    - 1st incubator (Eppendorf – Thermomixer comfort)
    - 2nd incubator (Eppendorf – ThermoStat plus – no. Z605190)
- Centrifuge
    - 1st centrifuge (NOVOLAB – Centrifuge 5430R – made in Geraardsbergen, Belgium)
    - 2nd centrifuge (Centrifuge 5430R – made in Hamburg, Germany)

After sample preparations, the InstaGene Matrix bottle was placed on the automatic stirrer to keep the beads in suspension. The substance contains beads which help to break open the cells and free the DNA. The top of a blue tip is cut off to avoid that the beads block the entry pathway. 200 µL from the InstaGene Matrix was added to each Eppendorf tube, which already contained the abdominal content of one bumblebee and vortexed manually. After this, 10 µL proteinase K was added, the samples were vortexed once more, put in the mini centrifuge for a few seconds and then placed in an incubator.

One automatically mixing and one static incubator were used. The samples were first incubated at 56°C for two hours, then at 97°C for 15 minutes. The samples were removed from the incubators between these two steps, because the machines had to warm up to obtain optimal temperature. For the static incubator, an extra step had to be performed. Each 15 minutes, the samples were vortexed manually to create motions similar to the moving incubator, which continuously spins at 600 rpm.

After incubation, the samples were centrifuged at 14,000 rpm for two minutes at room temperature (21°C). The beads and unwanted excess components can be separated from the supernatant as they form a pellet and dense emulsion at the bottom. The supernatant was transferred to a new Eppendorf tube and labelled correctly.

3.2.5.3 qPCR

Materials:

- 96-well plate (BIO RAD hard-shell PCR plates 96 well, thin wall – no. HSP9655 – made in USA)
- Seals (BIO RAD – microseal B adhesive sealer – no. MSB1001 – made in UK)
- qPCR machine 1 (BIO RAD – CFX96 Real-Time System Optics Module & C1000 Touch Thermal Cycler – no. 785BR08873 – made in Singapore (2012))
- qPCR machine 2 (BIO RAD – CFX96 Real-Time System Optics Module & C1000 Touch Thermal Cycler – no. 785BR14887 – made in Singapore (2016))
- 0.1 – 2.5 µL, 20 – 200 µL Pipettes (BRAND – Transferpette S – made in Germany)
- Tips
    - Yellow tips (Kima – Yellow tip – no. 18260 – made in Piove di Sacco, Italy)
    - White tips (Deltalab – 0.1-10 µL tip – no. 200024 – made in Barcelona, Spain)
- GoTaq qPCR Master Mix (Promega – no. A6002 – made in USA)
- Nuclease free water (Promega – Nuclease free water – no. P119E – made in Madison, USA)
- The forward and reverse primers sequences from Integrated DNA Technologies (IDT) to detect presence of any from the following genus (not limited to species) in the bumblebee gut:
    - *Apicy*stis
        - Neo Forward "5- CCAGCATGGAATAACATGTAAGG -3"
        - Neo Reverse "5- GACAGCTTCCAATCTCTAGTCG -3"
    - *Crithidia*
        - SE Forward "5- CTTTTGGTCGGTGGAGTGAT -3"
        - SE Reverse "5- GGACGTAATCGGCACAGTTT -3"
    - *Vairimorpha* (*Nosema*)
        - Nos Forward "5- TATGCCGACGATGTGATATG -3"
        - Nos Reverse "5- CACAGCATCCATTGAAAACG -3"
- Plate spinner (Labnet – MPS 1000 mini plate spinner)

In total 24 96-well plates were used for qPCR, where all 738 bumblebee samples and controls were tested for the presence of the three pathogens. Eight times, three identical 96-well plates were prepared. In each well, two µL of sample was applied. Each 96-well plate contained 94 bumblebee samples and one negative control (nuclease free water). One well was left open as we did not have a positive control.

While working on ice, the correct reaction mix was made by combining the calculated volumes of the following components:

- GoTaq qPCR mix (10 µL)
- Forward primer (1 µL)
- Reverse primer (1 µL)
- Nuclease free water (6 µL)

The values between brackets are the volumes used per sample. Three such reaction mixes were prepared, one per primer pair. 18 µL of mix was added to each well.

The plates were sealed and centrifuged in the plate spinner at low speed for 30 seconds. Then, the plate was transferred to the qPCR machine. The protocol which needs to be executed, is the same for all the primers, as they have the same characteristics. The "virus detection Biobest 57°C protocol" is selected in both qPCR machines which follows the curve as shown in ***Figure 3.6***. One run lasts approximately two hours. The SYBR green channel is measured in all 96 wells. The results were analysed using the "BIO-RAD CFX Manager" software.

qPCR is used to detect and amplify a small amount of target DNA. This involves: denaturation (DNA strands separate), annealing (primers bind to pathogen DNA) and extension (DNA strands are copied using DNA polymerase) as shown in ***Figure 3.6***.

Nuclease free water samples were used as negative controls. As no positive control was available in the beginning, a correct interpretation of the data was only possible after the qPCR cycles. As time was too limited, it was not possible to test positive samples twice or redo different plates.

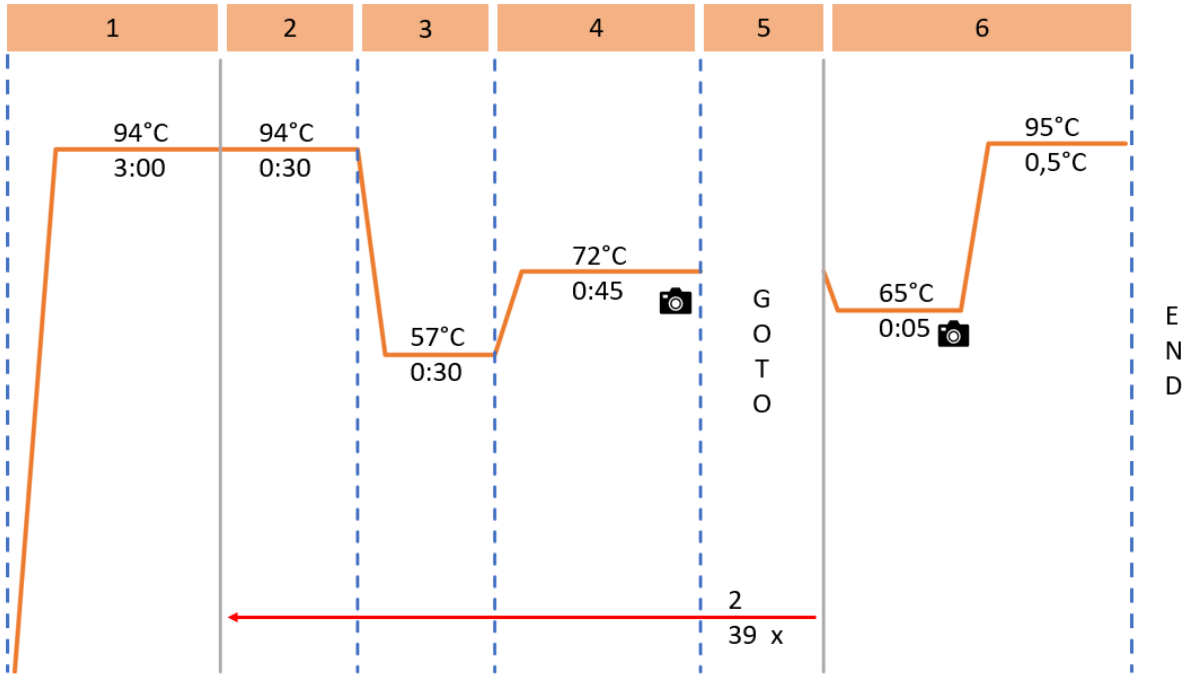

Figure 3.6: *Protocol followed by qPCR machine for the pathogen detection. Temperature (°C) is shown vertically, time horizontally (minutes:seconds). Different "phases" are indicated from one to six. Phase one (94°C – 3 minutes) is followed by 39 cycles of phases 2 – 3 – 4 and 5 to obtain the amplification curve. In phase six, the melting temperature is obtained. The whole run takes two hours. All 96 wells are measured using the SYBR green channel.*

Results were interpreted as being positive (Yes), negative (No) or inconclusive (Maybe). The evaluation of the qPCR results was done by looking at the shapes of the amplification curves, the Cq values, the melting temperature and corresponding peak. The temperature peak for each pathogen is different: the peak for *Apicystis* and *Vairimorpha* must be at 80°C, for *Crithidia* at 84°C.

The samples showing no DNA amplification during the qPCR cycles do not contain the targeted pathogens and are considered to be negative. Also samples with a wrong melting temperature were

excluded and placed in the negative group, as a wrong peak means that the sample is contaminated (e.g. because of the rough DNA extraction method) or that the forward and reverse primers bound each other instead of pathogen DNA.

The SYBR green baseline threshold was calculated automatically. The Cq (quantification cycle) value is the number of cycles at which the fluorescence first rises above the baseline threshold.

Bumblebees with an acceptable shape of the amplification curve, a correct melting temperature and Cq value ≤ 35 were placed in the positive group. An example can be seen in ***Figure 3.7***.

The samples with a correct melting temperature and an acceptable shape, but with Cq > 35 ≤ 39 may be positive, but might also be false positives. These results were all placed in the inconclusive group. All other samples below the threshold level with Cq "NA" or > 39 were placed in the negative group.

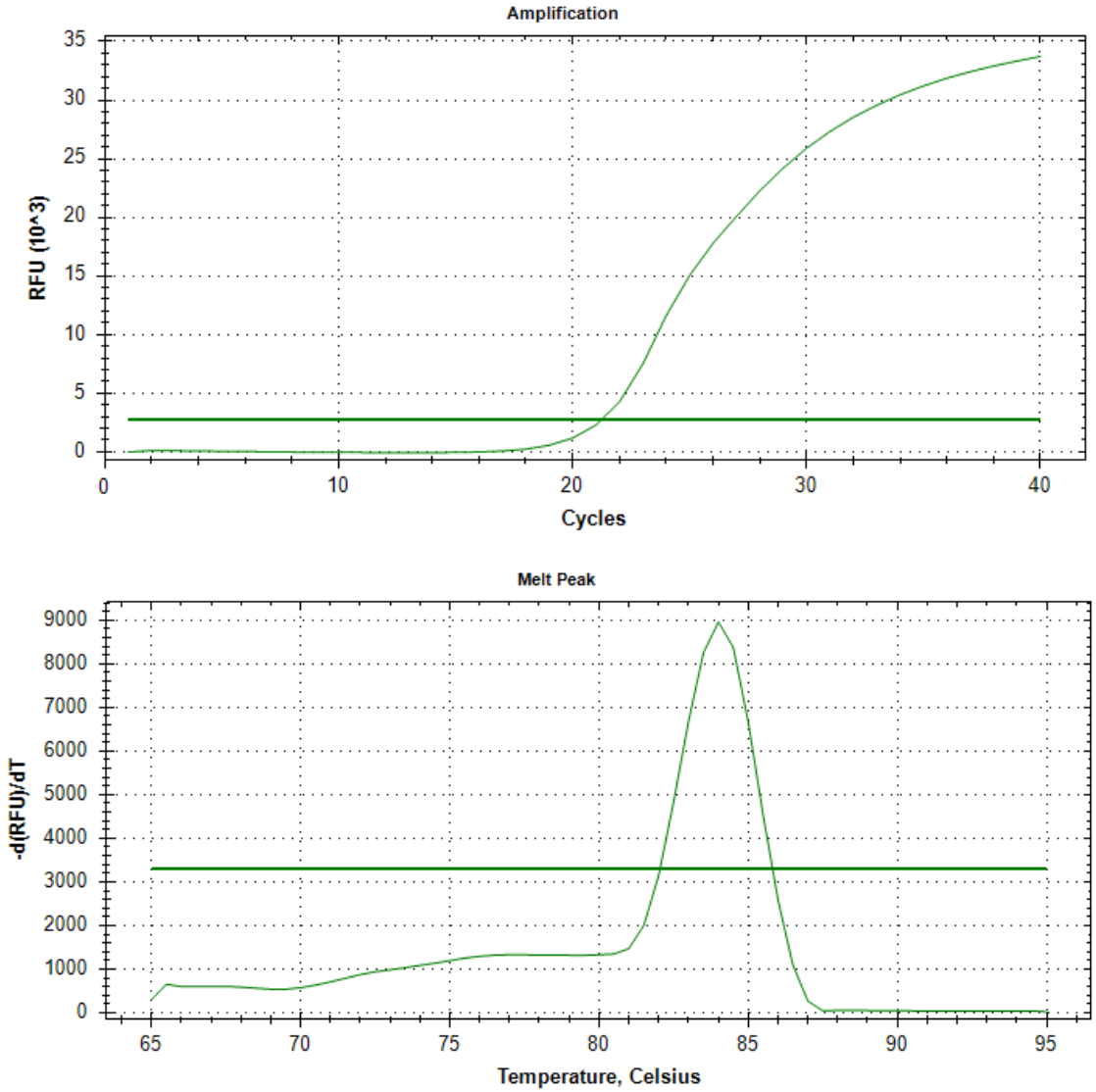

Figure 3.7: *Result of one sample obtained during own qPCR with primers (SE) detecting* Crithidia. *Top: Amplification curve with a sigmoidal shape (y-axis = relative fluorescence units (RFU), x-axis = # cycles). Bottom: Melting curve with correct melting peak at 84°C (y-axis = -d(RFU)/dT, x-axis = temperature (°C)).*

3.3 STATISTICAL ANALYSIS

3.3.1 Evaluation of pesticide and nutrition experiments

Survival curves, also known as Kaplan-Meier curves, were plotted, showing time on the x-axis and survival rate on the y-axis (Stel et al., 2011). The time refers to the period in days between the start of the experiment (placement in individual tubes containing sugar water with/without Spinosad) and the death of the bumblebees.

To determine whether location or pesticide concentration had an influence on the bumblebees' survival, a Log rank test was performed, comparing the curves between different groups (e.g. the agricultural and natural bumblebees or different pesticide concentrations).

The null hypothesis H0 is that both groups have identical curves. The goal is to prove that the alternative hypothesis H1, which states that there is a significant difference between the curves, is more likely to be true and to reject this H0. A p-value can be calculated and interpreted if this value is below or above the significance level which is set at 5% (0.05) at the beginning of the experimental set-up, together with all the goals and parameters that had to be tested. If the p-value ≥ 0.05, H0 cannot be rejected. If the p-value < 0.05, H0 is rejected and H1 is assumed to be true. For the Log rank test, the p-value was calculated based on the chi-squared (χ^2) distribution, since the Log rank statistic is equivalent to a χ^2 value.

With R Studio, curves can be plotted and statistically evaluated by loading the *survival* and *survminer* packages. Using *Surv*, *survfit*, *ggsurvplot* and *survdiff* commands and by indicating the right survival time, groups and events in the dataset, the curves can be plotted and the p-value calculated.

One may not forget that the Kaplan-Meier curve and analysis focusses on the entire survival curve, rather than on some smaller, possibly interesting parts of the curve (Rich et al., 2010).

3.3.2 Evaluation of temperature experiment and morphological data

Various methods can be used to test if the means of all measurements between the different places are significantly different. Several statistical tests and methods can be used to evaluate the data.

Some assumptions have to be met to obtain an interpretable result (Bevans, 2022).

- The data needs to be normally distributed. To evaluate this, a QQplot can be made with *qqnorm* and *qqline*, or a histogram, which has to result in a bell shaped, gaussian curve.
- The variances between the groups have to be similar (homoscedasticity). To evaluate this, a *leveneTest* is performed.
- The data of the sampled groups has to be independent of each other.
- Sampling must have been performed randomly.

Depending on the research question and available data, one has to select the best evaluation method. Each test or method has its advantages and disadvantages (Bevans, 2022).

In our experiments, generalised linear models (GLM) will be used. GLM and LM (linear models) are similar, but GLM is more flexible and can be used on non-normal data. GLM models can be fit to the data and since the function *glm* can handle binomial and poisson distributions, linear regression and logistic regression, it is easy to include different predictor variables and possible interactions (Casals et

al., 2014). The *summary* function will then give estimates, standard deviations and p-values. In the output of the summary function, an Akaike information criterion (AIC score) is shown, which can be used to compare different models and select the best one. The model with the lowest AIC score is preferred, as this regression model is able to fit the data in the best way. GLM models can be used to see what predictor variables have a high impact on the outcome, to execute risk analysis or make predictions.

3.3.3 Evaluation of influence of pathogens on previous experiments

To evaluate the influence of pathogens on the pesticide and nutrition experiments, Kaplan-Meier survival curves were made as with the pathogen and nutrition experiment statistical analysis. The Cox proportional hazard model *coxph* was used to calculate different p-values, after inspecting if the assumptions were met e.g. proportional hazard assumption using *cox.zph* (Bates et al., 2015). To evaluate the effect of pathogens on critical temperature and survival, generalised linear mixed models (GLMM) were created and compared with ANOVA (analysis of variance). The different models compared, consist of one full model M1, containing all parameters, and one basic model M0, which misses a variable of interest. In this way, one can test a lot of parameters, combinations and interactions to find the best model.

Generalised linear mixed models (GLMM) were used to evaluate the influence of pathogens on previous experiments. This was done in R using the *lme4* package and *glmer* (includes a link function to predict responses with non-gaussian distributions e.g. binomial distribution) and *lmer* functions (Bates et al., 2015).

In ***Appendix 4***, the basic R codes used for the statistical evaluations are shown.

4. RESULTS

4.1 PESTICIDE EXPERIMENT ONE

4.1.1 Observations pesticide experiment one

Once the experiment had started, the status of each bumblebee was evaluated at different time intervals (morning, noon, afternoon).

For the highest pesticide concentration P2, almost all bumblebees died within 24 hours. There was no difference between the natural and agricultural locations. This indicates that this concentration is harmful and lethal to all bumblebees. Since all the controls were still alive after 48 hours, there is a clear effect of the Spinosad concentration being present in the sugar water.

For the lower concentration P1, most of the bumblebees lived longer than the ones with the higher concentration P2. On average, P1 bumblebees could survive for two and a half days. From the P1 group, it seemed as if some agricultural bumblebees lived longer than the natural ones (see also statistical evaluation below). The P1 bumblebees died much faster than the controls, which again suggests an important effect of Spinosad on bumblebees and that, depending on the concentration, the survival time differs. Some controls died during the first four days, but most stayed alive for at least ten days, which was seen as the end of this pesticide experiment. After this date, some of the controls were used in the temperature experiments.

The observed effect is clear: Spinosad has a lethal effect and shortens the lifetime of bumblebees significantly when administered in a sugar water solution with doses of 40 µg/mL and 4 µg/mL.

4.1.2 Statistics pesticide experiment one

The time, survival period and event type were gathered from the pesticide experiments for statistical analysis. For all experiments, event value “one” marks the “death” of the bumblebee. Nearly all bumblebees died and got this value. Event value “zero” means the bumblebee stayed alive during the experiment. This is only true for the controls from the first pesticide experiment which survived for more than ten days, after which they were used in the temperature experiment. They are seen as censored events and have to be taken into account.

To see if there is a significant difference in the survival curves of bumblebees with **Spinosad concentrations P2 (40 µg/mL), P1 (4 µg/mL) and P0 (0 µg/mL)** two datasets were used, one containing all “L” bumblebees, the other all “N” bumblebees. “P2 *versus* P1”, “P2 *versus* P0”, “P1 *versus* P0” were plotted in ***Figure 4.1*** to see if there is a significant difference between the curves in each plot. Kaplan-Meier curves were made for “all agricultural” and for “all natural” bumblebees.

The observations made during the experiment are reflected and visualised in the curves. The survival rate of bumblebee groups clearly decreased if the pesticide concentration was higher. All p-values are significant (having all a p-value **<0.0001**, except one value of **0.004**, which are all smaller than the pre-set value $\alpha = 0.05$). All the calculated χ^2, df and p-values are shown in ***Appendix 3 (Table A3.1-A3.2)***.

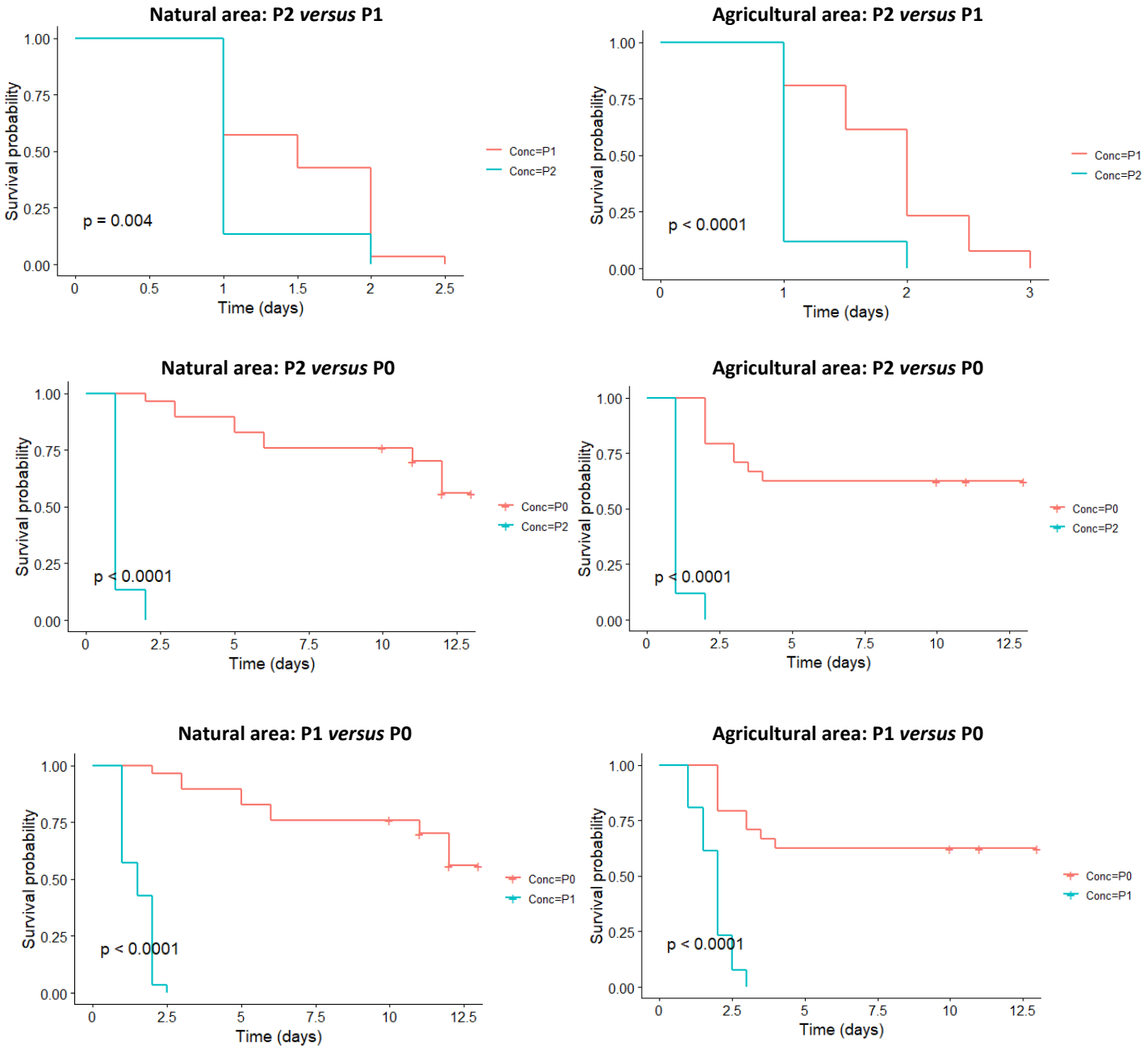

***Figure 4.1**: Kaplan-Meier curves, comparing concentrations of pesticide P0 (0 µg/mL), P1 (4 µg/mL) and P2 (40 µg/mL). All natural "N" bumblebees (left graphs) and all agricultural "L" bumblebees (right graphs).*

In the following, we investigate if there is a significant difference in the survival curves of **"N" and "L" bumblebees** with a certain pesticide concentration.

The difference between the N and L bumblebees receiving the highest concentration P2 in ***Figure 4.2*** is not significant (p-value = 0.88). Since nearly all of them died in the first 24 hours, this concentration is lethal and might be too high to make a distinguishing effect over time between the different groups.

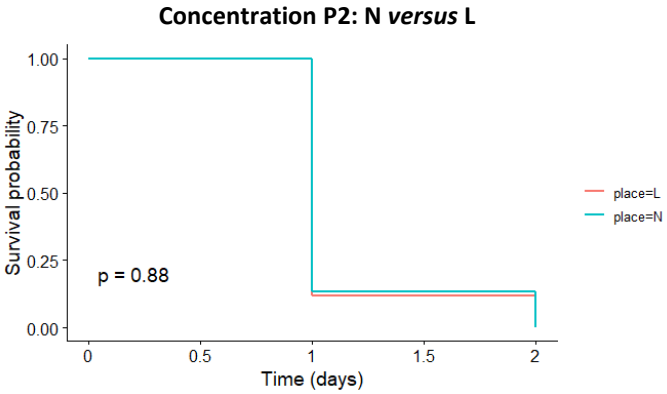

Figure 4.2: *Kaplan-Meier curves, comparing natural "N" and agricultural "L" bumblebees at fixed pesticide concentration P2 (40 µg/mL).*

The difference between the N and L bumblebees receiving concentration P1 is significant (p-value = **0.025**). As can be seen in ***Figure 4.3,*** the bumblebees coming from the natural areas die faster than the ones coming from agricultural areas, suggesting that bumblebees living near fields of agriculture linked with the use of pesticides, can better cope with or tolerate this pesticide concentration for a longer time.

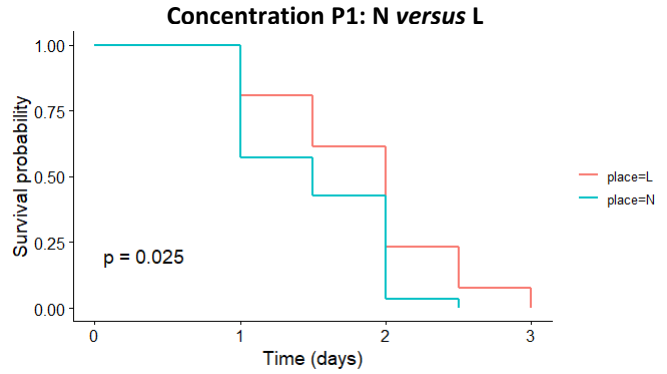

Figure 4.3: *Kaplan-Meier curves, comparing natural "N" and agricultural "L" bumblebees at fixed pesticide concentration P1 (4 µg/mL).*

Figure 4.4 shows that the controls P0 do not differ significantly from each other (p-value = 0.43), which is as expected. Some drops are seen in the plot for both groups in time, but they stay quite stable compared to the ones with a lethal dose. The bumblebees lived much longer compared to the P1 and P2 groups.

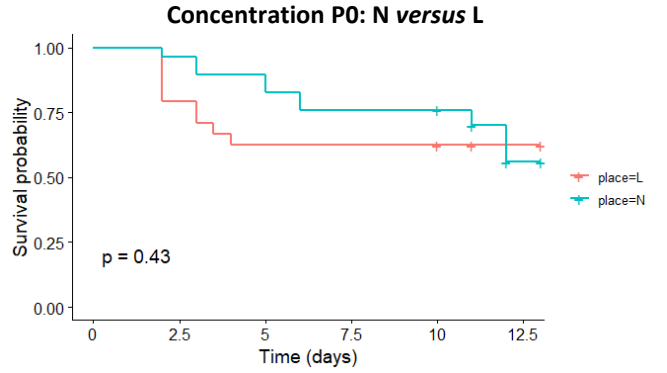

Figure 4.4: *Kaplan-Meier curves, comparing natural "N" and agricultural "L" bumblebees at fixed pesticide concentration P0 (0 µg/mL).*

4.2 PESTICIDE EXPERIMENT TWO

4.2.1 Observations pesticide experiment two

As all bumblebees with P1 and P2 concentrations died very quickly, a second pesticide experiment was set up, using the same evaluation procedure, but with lower Spinosad concentrations P3 and P4, diluted from the original P1 and P2 concentrations of the first pesticide experiment. The new concentrations P3 and P4 were tested with new controls P0.

The bumblebees' status was evaluated again at different times. In contrast to the first experiment, most bumblebees survived the first 24 hours.

In general, P4 bumblebees died faster compared to P3 and P0. The controls P0 died at a similar rate as the P3 bumblebees. No clear differences were observed between L or N bumblebees.

Since P3 and P0 bumblebees died at the same rate, it seems that the P3 concentration has no lethal effect on the bumblebees. However, since the controls died faster than expected, their death might be linked to another factor than the pesticide, making the interpretation more complex. A possible suggestion is that age influences our results, since the second experiment was conducted one week after the first pesticide experiment, using the same generation of bumblebees. These individuals were already older when starting the test. Research by Grund-Mueller et al. (2020) states that the type of diet influences lifespan, indicating that the bumblebee's longevity is increased when they are also fed pollen instead of giving sugar water (sucrose) only. This might also explain the fast death of our control bumblebees used in this experiment.

4.2.2 Statistics pesticide experiment two

Comparing **concentrations P4 (0.4 µg/mL), P3 (0.04 µg/mL) and P0 (0 µg/mL)**.

The same pipeline was followed to evaluate this experiment. Kaplan-Meier curves were plotted as seen in ***Figure 4.5***. All bumblebees got the "event" value one, as they all died while continuing the experiment. The (χ^2), df and p-values are shown in ***Appendix 3 (Table A3.3-A3.5)***.

When comparing P4 and P3, a trend in the graphs suggest a faster mortality for the P4 concentration for both N and L bumblebees, however the p-value was not significant.

The difference between P4 and P0 for the natural group was significant (p-value = **0.0033**), which suggests that this concentration still has a lethal effect. For agricultural bumblebees the difference between P4 and P0 was not significant, but as explained later, controls of agricultural bumblebees died fast making that this result cannot be trusted completely.

For P3 *versus* P0, no significant results were obtained, neither for N nor for L bumblebees. These results can indicate that the lowest pesticide concentration used (P3 = 0.04 µg/mL), has no significant impact on the survival time of the bumblebees and suggests that this pesticide concentration is neither lethal nor harmful when describing the effect on survival of the bumblebee individuals.

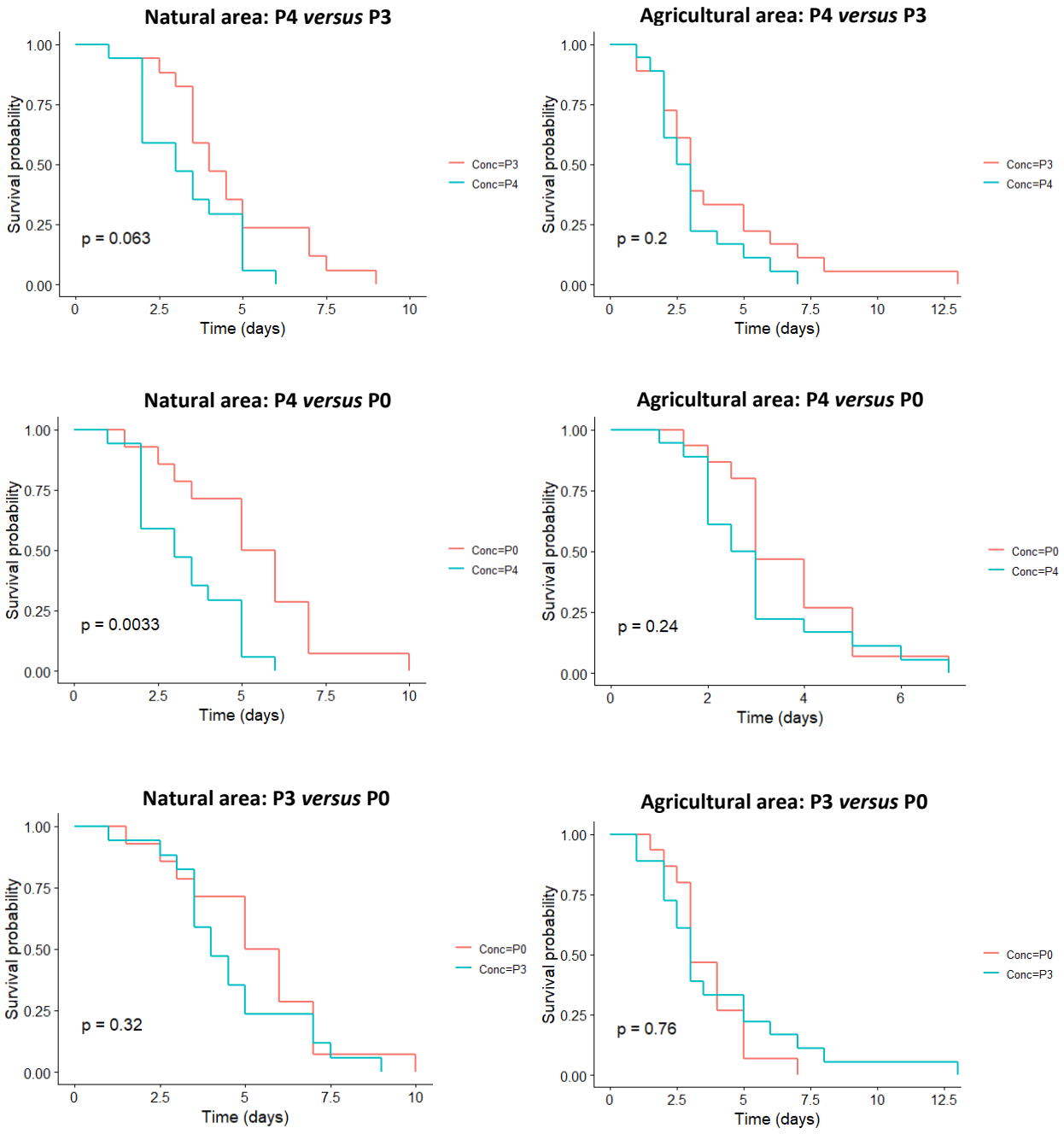

Figure 4.5: *Kaplan-Meier curves, comparing concentrations of pesticide P0 (0 µg/mL), P3 (0.04 µg/mL) and P4 (0.4 µg/mL). All "N" bumblebees (left graphs) and all "L" bumblebees (right graphs).*

Comparing **N and L** bumblebees:

The difference between the N and L bumblebees receiving P4 in ***Figure 4.6*** is not significant (p-value = 0.7).

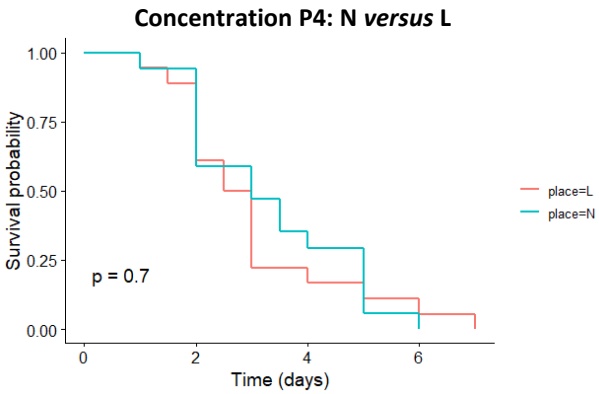

Figure 4.6: *Kaplan-Meier curves, comparing natural "N" and agricultural "L" bumblebees at fixed pesticide concentration P4 (0.4 µg/mL).*

The difference between the N and L bumblebees receiving P3 in ***Figure 4.7*** is also not significant (p-value = 0.51).

No difference seems to be present between the L and N bumblebees when P3 and P4 are applied.

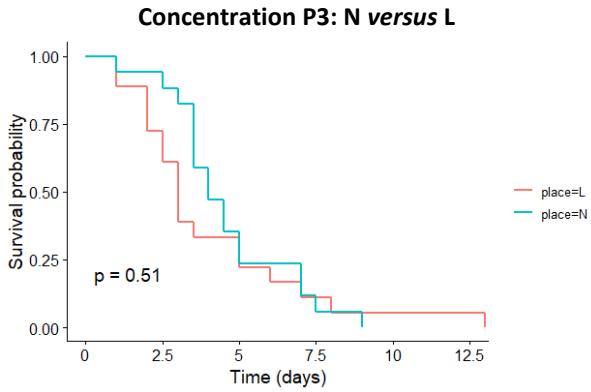

Figure 4.7: *Kaplan-Meier curves, comparing natural "N" and agricultural "L" bumblebees at fixed pesticide concentration P3 (0.04 µg/mL).*

The difference between the N and L bumblebees receiving P0 in ***Figure 4.8*** is significant (p-value = **0.019**). This is not as expected. The controls should not give any difference to make proper conclusions and have trustworthy results. This is not the case.

It is seen that the agricultural bumblebees die faster. As these were the controls, this is not because of the added pesticide concentration. Thus, we assume this is mostly linked to age, but it can also be due to the presence of a pathogen or a location specific effect.

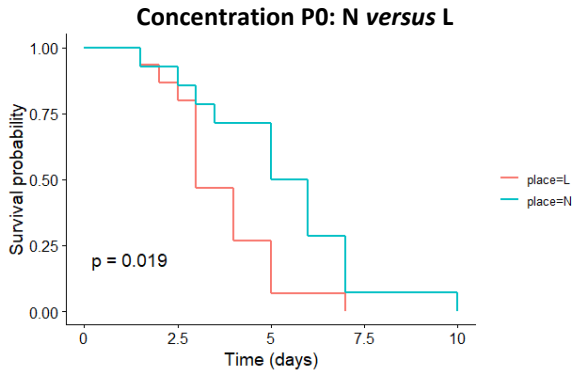

Figure 4.8: *Kaplan-Meier curves, comparing natural "N" and agricultural "L" bumblebees at fixed pesticide concentration P0 (0 µg/mL).*

No definite conclusions can be made, but the data can be analysed to see if there are interesting/remarkable events. When looking deeper into the results and comparing different agricultural locations, there is a noticeable difference. During the second pesticide experiment, in general, the bumblebees of the L1 location died significantly faster compared to L2 (p-value = **0.0049**) and L3 (p-value = **0.046**). L2 and L3 are not significantly different (p-value = 0.48).

When removing all L1 bumblebees and comparing the remaining L groups (L2 and L3) with the whole N group, the controls between N and L are not significant anymore (p-value = 0.082). Also the differences between L and N for P3 (p-value = 0.92) and P4 (p-value = 0.72) stay non-significant.

The significant effect of the P4 concentration (0.4 µg/mL) on survival was clear for the natural bumblebees. This was not the case for the agricultural bumblebees. Two possible interpretations can be made. Firstly, since the survival time of the P0 group of agricultural bumblebees was not as expected and L and N survival did not significantly differ when receiving the P4 concentration, this concentration might still be lethal for bumblebees from both places. Secondly, the concentration might be lethal for natural bumblebees who are less resistant (as was also observed for P1 during the first pesticide experiment) and agricultural bumblebees can handle this concentration better. However, more research will be needed to draw certain conclusions.

The P3 concentration (0.04 µg/mL) has no lethal effects on bumblebees and no differences are noted between L and N bumblebees.

4.3 NUTRITION EXPERIMENT

4.3.1 Observations nutrition experiment

The S1 (25%) and S0 (50%) groups were evaluated in the same way as for the pesticide experiments.

The survival rates for the S1 and S0 groups, as well as for the bumblebees from the L and N locations looked similar. However, it was clear that the bumblebees feeding on the reduced sugar water solution, drunk more compared to the control group. They survived by drinking more of the lower sugar solution concentration, so that in fact the total sugar intake was rather similar for both groups.

We assume that all bumblebees died because of age and not because of the sugar concentrations used in the experiment.

It was remarkable that the bumblebees from the nutrition experiment (both S0 and S1) drunk much more compared to the bumblebees which had sugar water containing pesticides. This might suggest that the pesticide has a repelling effect. The bumblebee “senses” there is something in the solution and only consumes a bit when really needed.

4.3.2 Statistics nutrition experiment

The sugar concentrations S1 (25%) and S0 (50%, control) were compared for both the agricultural and natural area bumblebee groups. The χ^2, df and p-values are noted in ***Appendix 3 (Table A3.6-A3.7)***. The different concentrations do not seem to have a high impact on survival capacity as long as food is present. As seen in ***Figure 4.9***, both the bumblebees from N (p-value = 0.83) and L (p-value = 0.052) locations can handle reduced sugar concentrations. Mortality does not significantly increase when sugar concentration is reduced from 50% to 25%. It might be interesting to perform this experiment again in future research testing more and lower concentrations, as the p-value for the “L” group was very close to the 0.05 significant threshold.

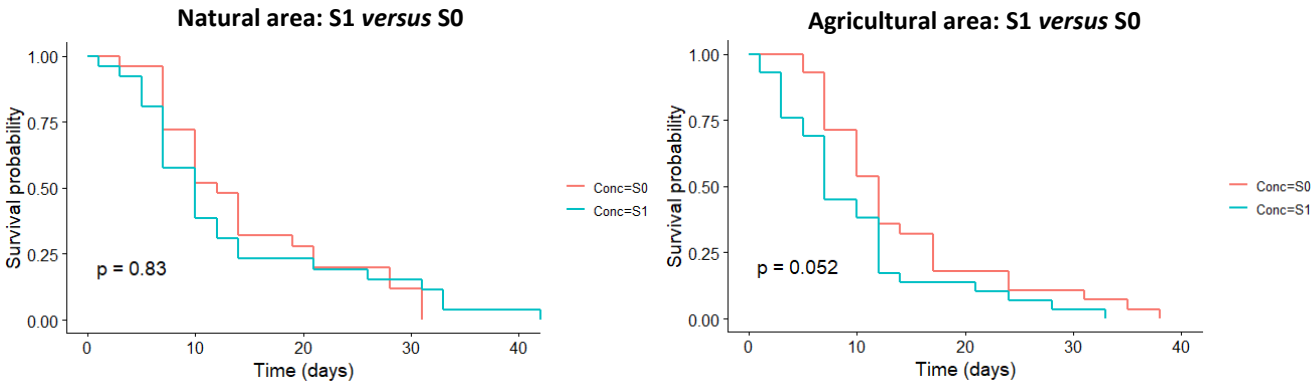

Figure 4.9: *Kaplan-Meier curves, comparing S1 (25%) and S0 (50%) sugar concentrations. All "N" (left) and all "L" (right) bumblebees.*

For both tested sugar concentrations in ***Figure 4.10***, there are no significant differences for the L and N bumblebees. For the control group (p-value = 0.92), this is as expected and makes the conclusions trustworthy. Also for S1, the L and N do not vary significantly (p-value = 0.18).

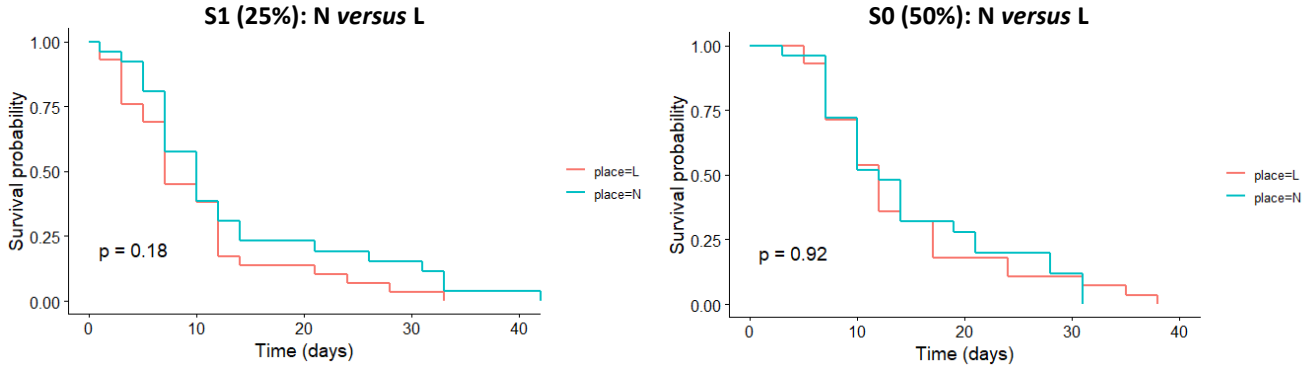

Figure 4.10: *Kaplan-Meier curves, comparing natural "N" and agricultural "L" bumblebees at a fixed sugar concentration S1 (25%) (left) and S0 (50%) (right).*

To conclude: the reduced sugar concentration did not influence the survival rate and no difference between the L and N bumblebees was found as all p-values resulted >0.05 (5%).

The **amount of mL** drunk by the bumblebees was monitored to confirm the observations. This value was divided by the survival time (in days), to get the "amount of mL per day" that can be analysed. In ***Table 4.1,*** the mean values are shown per day. In line with previous observations, more mL was drunk by the S1 individuals compared to S0, indicating that the higher the sugar concentration, the lower the volume needed. There was no significant difference between L and N bumblebees. When using GLM models, where the data meets all the assumptions, the same conclusions were made. The mL drunk was significantly different for S0 and S1 bumblebees (p-value = **0.00122**), whereas for the drinking of L and N bumblebees, no significant difference was noted for both S0 or S1 (p-value = 0.984 and p-value = 0.879 respectively).

Table 4.1: *Average volume (mL) sugar water (S0 = 50% and S1= 25%) drunk by the bumblebees during the nutrition experiment.*

SUGAR CONCENTRATION	LOCATION	VOLUME # mL / day
S0	L	0.31
	N	0.31
S1	L	0.40
	N	0.39

4.4 TEMPERATURE EXPERIMENT

4.4.1 Observations temperature experiment

After the temperature cycles, the video recordings were evaluated, which included: determining the exact time at which the bumblebees lose their muscle control or fall and the corresponding cold and warm environmental temperature. Minimum, maximum and mean values were all gathered. Since not all videos recorded the complete cycle due to problems with the capacity of the processing computer, the amount of samples and data is limited. However, the available data can still be analysed.

4.4.2 Statistics temperature experiment

For measuring cold tolerance (CT_{min}), the mean value of the environmental temperature was used since the maximum value would correspond to the higher temperature of the bumblebee itself. For measuring heat tolerance (CT_{max}), the minimum value of the environment is of interest.

The critical temperature at which the bumblebees lose muscle control and fall over, is measured. Temperature means are calculated and shown in ***Table 4.2*** for all agricultural (L), all natural (N) and for all bumblebees together. Out of these results, one can assume that the mean estimated critical temperatures of *B. pascuorum* are approximately $CT_{min} \approx 0.6$ °C and $CT_{max} \approx 47.7$ °C.

Table 4.2: *Critical temperatures (CT_{min} and CT_{max}) of* B. pascuorum.

	ALL L	ALL N	ALL BUMBLEBEES
CT_{min} (°C)	0.6	0.5	0.6
CT_{max} (°C)	47.8	47.5	47.7

Since the bumblebee size influences its capacity to maintain or process warmth, the temperature value of each individual is divided by the average length of the left and right marginal wing cell. This results in a value which is not very informative itself, but can be used to compare different groups of bumblebees to see if there is a significant difference.

To evaluate if there is a significant difference between the critical temperatures for the agricultural and natural bumblebees, GLM were made. Example code is shown in ***Appendix 4 (Textbox A4.1)***.

All values were non-significant (all the p-values were > 0.05) for both the cold and hot periods. Also after relevelling (changing the reference factor), differences were non-significant.

We can conclude that the average critical temperatures of L and N *B. pascuorum* in this experiment are the same. This is as expected. Since all bumblebees were sampled in Belgium at a relatively small geographical scale, where climate and temperature are similar, the bumblebees do not have to adapt to extremely different temperatures.

4.5 MORPHOLOGICAL EVALUATION

4.5.1 Observations morphology

During the making of the morphology sheets, there was a clear visual difference between the mass and size of the female and male bumblebees at each location. The males were larger than the females. When comparing locations, it was noticeable that L2 had a lot of big female bumblebees, whereas female bumblebees at N3 were very small.

The size of the wings and legs can be linked to the mass and total length of the bumblebee. Smaller bumblebees had smaller wings and smaller legs, the opposite was true for the bigger bumblebees.

All measurements were evaluated in R Studio. The females and males were split up to be evaluated separately and different location groups were made. Averages of the radial cell of the left and right forewing, the mass and the six leg compartments were made. The average values followed the observations described above.

4.5.2 Statistics morphology

Information is available from 738 bumblebees, of which 644 females (636 workers, 8 queens) and 94 males.

Figure 4.11 shows a histogram with only the female bumblebees' weight, which has clear outliers at the right tail. After removing the extreme values, the histogram seems normally distributed. The bumblebees with a mass above 229 mg are assumed to be (future) queens. These eight biggest values were removed, as they were much bigger compared to the average weight of all the other female bumblebees. The labels of the queen bumblebees, removed from the morphological evaluation, are: L3G6, L3G23, L3F22, L2E5, L3G8, L3F10, L3F21 and L3F26. These individuals are all from agricultural locations (all L3 except 1), but since their mass is also much higher than the others from that location, this is not seen as a specific location trait, but merely from the fact that they are from another caste. The mean of all variables of interest were calculated for the male drones and female workers (without queens) for all L and all N locations and for all the bumblebees of the same sex together, as shown in ***Table 4.3***.

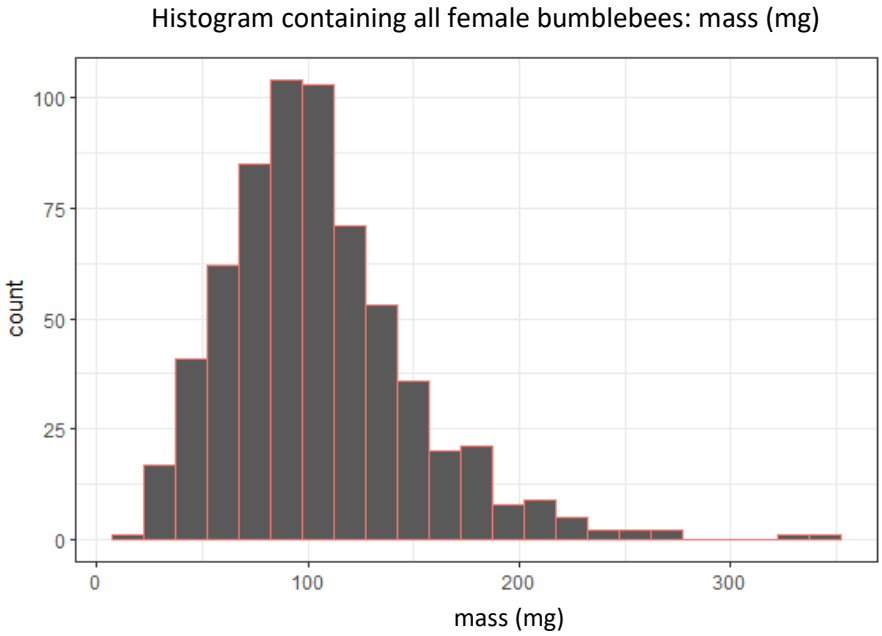

Figure 4.11: *Histogram showing the mass (mg) of all female bumblebees. Outliers with a high mass can be seen at the right, these are assumed to be queens. (y-axis = count, x-axis = mass (mg)).*

Our outcome variable of interest is a quantitative measurement: mass, length of radial cell, parts of the left middle leg. The predictor variable is the place (L or N), where the bumblebees originate from and in addition their sampling location (L1, L2, L3, N1, N2, N3). The means from the outcome variables will be compared between the L and N groups. Different models can be made, examples are given in ***Appendix 4 (Textbox A4.2)***.

Table 4.3: *Averages of all morphological measurements. Mass, length of radial cells, six leg compartments of the drone and worker bumblebees.*

	ALL L		ALL N		ALL BUMBLEBEES	
	Male	Female	Male	Female	Male	Female
Mass (mg)	127.68	105.09	122.11	99.84	125.49	102.71
Left radial cell (mm)	3.12	2.95	3.07	2.79	3.10	2.88
Right radial cell (mm)	3.11	2.96	3.09	2.81	3.10	2.89
Coxa (mm)	1.92	1.89	1.85	1.87	1.89	1.88
Trochanter (mm)	1.29	1.33	1.19	1.37	1.25	1.35
Femur (mm)	2.93	2.93	3.03	2.88	2.97	2.91
Tibia (mm)	2.81	2.80	2.86	2.74	2.83	2.77
Metatarsus (mm)	2.46	2.41	2.55	2.32	2.49	2.37
Tarsus (mm)	2.20	2.01	2.21	2.00	2.20	2.00

The sex (male/female) is a significant predictor of the mass (p-value = **3.48e-07**). Since there is a huge difference, two separate datasets are made: one for workers, one for drones. Otherwise, the other predictor variables may be overlooked.

First of all, the place (natural or agricultural area) is evaluated. The mass, the average length of the left and right marginal wing cells and the average length of the six leg compartments of L and N bumblebees are compared. For the females, these comparisons resulted in non-significant p-values, except for the length of the left and right marginal wing cells. For the males, all p-values were non-significant. Thus, we might assume that there is no significant difference in morphology between same sex bumblebees from natural and agricultural areas in general.

When looking at the specific locations (L1, L2, L3, N1, N2 and N3), highly varying results are seen and no conclusions can be drawn. For the females, no recurring patterns could be seen. The measurement variables (coxa, mass, etc.) for the different locations sometimes resulted in significant and sometimes non-significant results. When changing the reference to which all locations are compared, the results changed. So no clear conclusions can be made. One thing worth mentioning is that for most measurement variables, L3 and N3 seemed to be significantly different from the other locations with which they were compared, which is in line with the data obtained from the averages calculated. For males, most results were non-significant. Only L3 was significantly different in some cases.

4.6 PATHOGEN DETECTION

4.6.1 Observations pathogen detection

As our goal was to examine the differences between agricultural and natural bumblebees under various stressors, we simulated real life situations to get extrapolatable results. The pathogen factor is inevitable, as the bumblebees live and roam freely. Incorporating pathogens in this experiment is therefore very interesting.

4.6.1.1 Unexpected finding: the conopid fly

During the dissection and extraction of the bumblebees' abdominal contents, it was remarkable that a lot of the bumblebees which had died before they could be used in any of the experiments, carried a "red pupa" inside their abdomen, which looks similar to the photos and description in literature, indicating the presence of a parasitic conopid fly. In ***Figure 4.12***, one of the pupas found in our bumblebee dissections is shown.

Also, the appearance of the tissues and gut inside the bumblebees used in the different experiments varied significantly. For the individuals which received pesticides (groups P1, P2, P3 and P4), the extracted substance looked dark (brown/black), whereas for all the specimens from the temperature or nutrition experiments, as well as for the control groups of the pesticide experiments (P0), the inside looked white/yellowish.

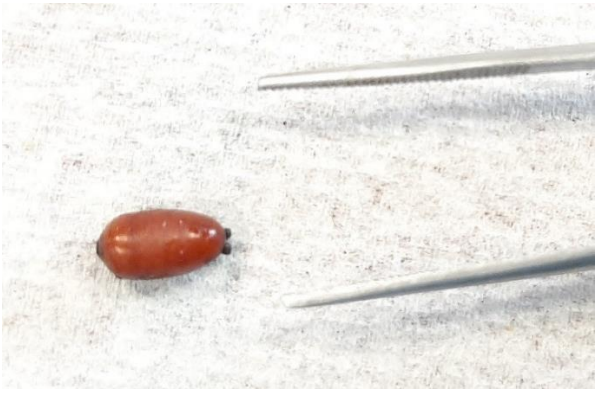

Figure 4.12: *Own photo of a conopid fly pupa extracted from bumblebee abdomen.*

4.6.1.2 Presence of pathogens

During the pesticide, nutrition and temperature experiments it was not possible to distinguish infected from non-infected bumblebees. The presence of the conopid fly parasite was determined visually while dissecting the abdomen of all 738 bumblebees. The abdomen contents were used to extract DNA on which qPCRs were performed to detect the presence of pathogens, i.e. *Apicystis*, *Crithidia* and *Vairimorpha* (*Nosema*). The pathogen data of two individual bumblebees, L1C12 and L1I29, was lost during the qPCR experiment, so these two were not included in the evaluation of the pathogen detection. ***Table 4.4*** shows the incidence of the different pathogens in all 736 *B. pascuorum*. A more detailed analysis of the influence of these pathogens on the experiments, as well as the presence of multiple pathogens in the same bumblebee will follow.

Table 4.4: *Numbers and percentages of bumblebees that tested positive/negative/inconclusive for the different pathogens.*

PATHOGEN	APICYSTIS			CRITHIDIA			VAIRIMORPHA			CONOPID FLY	
INFECTED	Yes	Maybe	No	Yes	Maybe	No	Yes	Maybe	No	Yes	No
TOTAL #	297	79	360	153	76	507	103	67	566	89	647
TOTAL %	40.4	10.7	48.9	20.8	10.3	68.9	14.0	9.1	76.9	12.1	87.9

The pathogens *Apicystis*, *Crithidia* and *Vairimorpha* have an incidence of 40.4%, 20.8% and 14.0%.

The presence of the conopid fly was detected in 12.1% of the bumblebees. It is safe to say that the presence of the conopid fly did not interfere with the results of our experiments as the bumblebees infected with this parasite all died before they could be used in the experiments (except one, which died after three days in the second pesticide experiment as a control). The fast death of all diseased individuals indicates the severe mortality when this parasite is present.

4.6.1.3 Presence of pathogens in agricultural (L) and more natural (N) areas

The incidence of pathogens for L (410 bumblebees) and N (326 bumblebees) was determined as shown in ***Table 4.5***.

Table 4.5: *Numbers and percentages of L and N bumblebees that tested positive/negative/inconclusive for the different pathogens.*

PATHOGEN	APICYSTIS			CRITHIDIA			VAIRIMORPHA			CONOPID FLY	
INFECTED	Yes	Maybe	No	Yes	Maybe	No	Yes	Maybe	No	Yes	No
All L #	149	36	225	74	46	290	52	29	329	64	346
All L %	36.3	8.8	54.9	18.1	11.2	70.7	12.7	7.1	80.2	15.6	84.4
All N #	148	43	135	79	30	217	51	38	237	25	301
All N %	45.4	13.2	41.4	24.2	9.2	66.6	15.6	11.7	72.7	7.7	92.3

The above results indicate that *Apicystis*, *Crithidia* and *Vairimorpha* are more present in the natural locations than in the agricultural locations. The reverse is true for the presence of the conopid fly.

4.6.1.4 Presence of pathogens per location and batch

The number of bumblebees collected at the different locations L1-L2-L3-N1-N2-N3 amounts to 148-127-135-97-133-96 respectively. Since there were different sampling moments in time, we analysed if there was a clear difference between the different locations or between the different batches. ***Table 4.6*** shows the incidence of pathogens in bumblebees (%) at different locations, containing some interesting results. ***Appendix 6 (Table A6.1)*** shows the incidence (#) of pathogens in bumblebees.

All pathogens are observed at least once at each location. *Apicystis* is present at all locations for all different batches. In most cases (except N3), *Apicystis* incidence is lower for the last batches caught, compared to the first batches. For *Crithidia*, the same holds, except for N2 and N3. For *Vairimorpha* this holds except for N2. Bumblebees are infected much more with *Apicystis* and *Crithidia* than with *Vairimorpha* and the conopid fly.

When looking into detail at the results for the conopid fly, locations N2 and N3 are rarely infected with sometimes even no diseased bumblebees. All locations show a decline in infected individuals over time, which might indicate that the conopid fly is more active during the summer months and less in September.

Table 4.6: *Incidence of different pathogens (%) in bumblebees from different locations (L1, L2, L3, N1, N2 and N3), collected in batches on various dates.*

LOCATION	BATCH	# BUMBLEBEES	APICYSTIS %			CRITHIDIA %			VAIRIMORPHA %			CONOPID FLY %	
			Yes	Maybe	No	Yes	Maybe	No	Yes	Maybe	No	Yes	No
L1	1 (04/08)	73	65.8	2.7	31.5	8.2	12.3	79.5	9.6	5.5	84.9	32.9	67.1
	2 (13/08)	46	21.7	10.9	67.4	8.7	15.2	76.1	15.2	0.0	84.8	19.6	80.4
	3 (21/08)	29	24.1	10.4	65.5	0.0	10.3	89.7	3.4	10.4	86.2	3.4	96.6
L2	1 (05/08)	71	39.4	8.5	52.1	40.8	11.3	47.9	12.7	5.6	81.7	23.9	76.1
	2 (21/08)	27	29.6	7.4	63.0	14.8	18.5	66.7	25.9	22.2	51.9	3.7	96.3
	3 (21/09)	29	20.7	0.0	79.3	17.2	6.9	75.9	3.4	20.7	75.9	0.0	100.0
L3	1 (09/08)	75	36.0	12.0	52.0	22.7	5.3	72.0	21.3	2.7	76.0	12.0	88.0
	2 (21/08)	29	20.7	10.3	69.0	17.2	3.4	79.3	10.3	6.9	82.8	10.3	89.7
	3 (21/09)	31	29.0	19.4	51.6	12.9	22.6	64.5	3.2	6.5	90.3	0.0	100.0
N1	1 (07/08)	70	54.3	5.7	40.0	22.9	12.9	64.3	22.9	8.5	68.6	14.3	85.7
	2 (21/08)	27	51.9	3.7	44.4	14.8	3.7	81.5	3.7	11.1	85.2	11.1	88.9
N2	1 (08/08)	65	53.8	10.8	35.4	32.3	12.3	55.4	16.9	7.7	75.4	12.3	87.7
	2 (23/08)	30	50.0	13.3	36.7	0.0	3.3	96.7	3.3	6.7	90.0	6.7	93.3
	3 (22/09)	38	42.1	13.2	44.7	47.4	15.8	36.8	18.4	23.7	57.9	0.0	100.0
N3	1 (10/08)	59	35.6	22.0	42.4	22.0	1.7	76.3	16.9	11.9	71.2	3.4	96.6
	2 (21/08)	19	10.5	36.9	52.6	0.0	0.0	100.0	15.8	21.0	63.2	0.0	100.0
	3 (21/09)	18	38.9	11.1	50.0	38.9	22.2	38.9	11.1	11.1	77.8	0.0	100.0

4.6.1.5 Presence of pathogens per bumblebee

When focusing on *Apicystis*, *Crithidia* and *Vairimorpha*, 211 bumblebees were healthy and free of pathogens, 237 were infected with one pathogen, 84 with two pathogens and 12 having all three. This corresponds to 38.8%, 43.6%, 15.4% and 2.2% respectively. The remaining 192 bumblebees all showed at least one inconclusive result and are therefore not taken up in this evaluation.

4.6.1.6 Presence of pathogens in male and female bumblebees

The incidence of pathogens in females (workers and queens) and males (drones) was also compared. The percentages are shown in ***Table 4.7***.

Table 4.7: *Incidence of different pathogens (%) in male and female bumblebees from L and N areas.*

PATHOGEN			APICYSTIS %			CRITHIDIA %			VAIRIMORPHA %			CONOPID FLY %	
INFECTED		#	Yes	Maybe	No	Yes	Maybe	No	Yes	Maybe	No	Yes	No
MALE	Total	94	42.5	12.8	44.7	18.1	10.6	71.3	12.8	12.8	74.4	6.4	93.6
	All L	57	40.3	8.8	50.9	19.3	10.5	70.2	14.0	7.0	79.0	8.8	91.2
	All N	37	46.0	18.9	35.1	16.2	10.8	73.0	10.8	21.6	67.6	2.7	97.3
FEMALE	Total	642	40.0	10.5	49.5	21.2	10.3	68.5	14.2	8.6	77.2	12.9	87.1
	All L	353	35.7	8.8	55.5	17.9	11.3	70.8	12.5	7.1	80.4	16.7	83.3
	All N	289	45.3	12.5	42.2	25.3	9.0	65.7	16.3	10.4	73.4	8.3	91.7

No extreme variations nor specific patterns were observed. However, workers seem to be infected twice as much with the conopid fly than males. This might be related to the specific tasks in the colony. Males only have reproduction as a goal and do not need to forage in the area to collect food for the colony as workers do. Our data only includes male and female foraging bumblebees collected outside the nests. To obtain a complete view when comparing castes, females inside the nest (nesters) should also be screened, as these should have a lower incidence and lower risk to get infected with the conopid fly.

When evaluating the bumblebees' morphology, we excluded the queens. However, since queens do not have the same role in the colony and have different tasks, we took a closer look at these individuals for the pathogen detection. None of the eight queens were infected with *Crithidia* or *Vairimorpha* pathogens, nor with the conopid fly parasite. Three were infected with *Apicystis*.

4.6.1.7 Influences on survival time

The following evaluation uses a dataset containing non-infected bumblebees and bumblebees infected with at least one pathogen, to detect possible influences of pathogens on the survival time or critical temperatures of bumblebees linked to the pesticide, nutrition and temperature experiments.

Both infected and non-infected individuals were present in all experiments, making that if pathogens would have had an effect, the experiments should have been influenced more or less in the same way.

B. pascuorum used in the experiments that were infected with *Apicystis*, *Crithidia* or *Vairimorpha* pathogens, were expected to die earlier than the healthy ones which would be more resistant to stress factors. However, when evaluating the pesticide one, pesticide two and nutrition experiments separately by making a general boxplot with infected *versus* healthy individuals as seen in ***Figure 4.13***, no real differences in survival time are seen. There is no clear indication that pathogens interfered with the experiments and we expect most of our following evaluations to be non-significant.

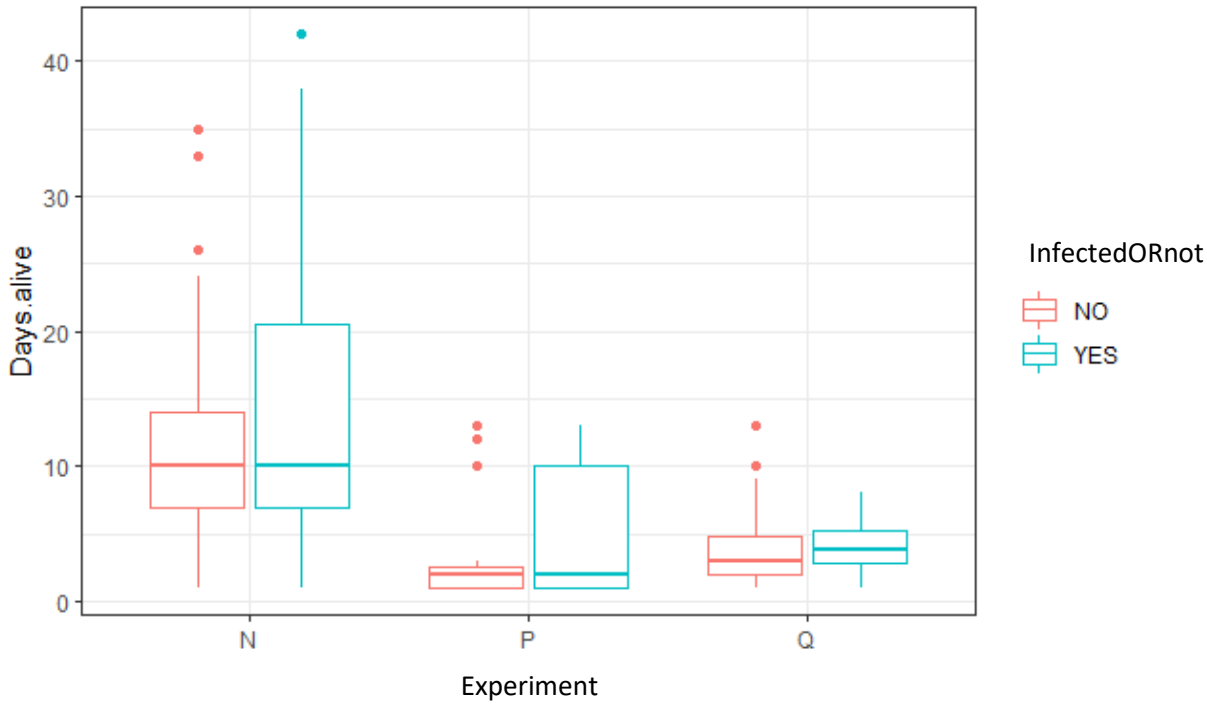

Figure 4.13: *Boxplot with survival time of infected versus non-infected bumblebees during the different experiments. Plotted with function geom_boxplot and its default settings. N (nutrition), P (pesticide one) and Q (pesticide two) experiments.*

4.6.1.8 Influence of pathogens on the control group of pesticide experiment two

As the controls of the second pesticide experiment died extremely fast, we took a closer look at this group. However, pathogen presence did not have an effect on the survival time of this group. The reason for the fast death is probably linked to age.

4.6.2 Statistics pathogen detection

Kaplan-Meier curves and GLLM models were made to statistically evaluate the effect of pathogens on our data. The basic code is shown in ***Appendix 4 (Textbox A4.3* and *A4.4)***.

4.6.2.1 Presence of pathogens in bumblebees from different places (L/N) and batches

A dataset containing non-infected bumblebees and bumblebees infected with at least one pathogen was used to compare different models with ANOVA, with the aim to evaluate if the variables “place” and “batch” are significant predictors for the presence of pathogens (which is indicated as a binomial response variable).

This resulted in **place** not being a significant predictor for the presence of any of the pathogens, with the following values: infected (p-value = 0.07406, χ^2 = 3.1907, df = 1), *Apicystis* (p-value = 0.09938, χ^2 = 2.7155, df = 1), *Crithidia* (p-value = 0.3568, χ^2 = 0.849, df = 1*), Vairimorpha (*p-value = 0.1675, χ^2 = 1.9054, df = 1).

The **batch** variable was a significant predictor for the presence of most pathogens, with the following values: infected (p-value = **2.849e-07**, χ^2 = 30.142, df = 2), *Apicystis* (p-value = **0.0001439**, χ^2 = 17.693, df = 2), *Crithidia* (p-value = **0.0001533**, χ^2 = 17.567, df = 2), *Vairimorpha* (p-value = 0.1181, χ^2 = 4.2722, df = 2).

We can therefore assume that the presence of pathogens varies for the different batches as seen in ***Table 4.6***, indicating that there was a higher prevalence of pathogens in the first batches collected in the beginning/middle of August, compared to the second and third batches collected at the end of August and September.

4.6.2.2 Influence of pathogens on pesticide/nutrition experiments

Using the qPCR results, pathogens can be included in our analyses. Kaplan-Meier curves were plotted to see if pathogens have an effect on the survival of the agricultural and natural bumblebees during the pesticide and nutrition experiments. All obtained p-values are shown in ***Appendix 3 (Table A3.8)***. Most p-values are not significant, which was as expected and in line with the boxplot ***(Figure 4.12)*** made, which indicated that the presence of pathogens did not influence our experiments.

Only a few significant values were obtained as described below:

- The statistical evaluation of bumblebees from the first pesticide experiment with a concentration of 4 µg/mL (group P1) showed a significant difference in survival between bumblebees from agricultural (L) and natural (N) areas. This significant difference for place (L/N) was confirmed when the influence of *Apicystis* and *Crithidia* was evaluated, obtaining p-values of **0.033** and **0.0298** respectively. Moreover, the presence of these pathogens did not have a significant effect on the survival of these individuals.

- The statistical evaluation of bumblebees from the second pesticide experiment with a concentration of 0 µg/mL (control group P0) showed a significant difference in survival between bumblebees from agricultural (L) and natural (N) areas. This was not as expected. When evaluating the influence of *Apicystis*, *Crithidia* and *Vairimorpha*, when comparing L *versus* N, p-values of **0.0232**, **0.019** and **0.0263** were obtained respectively, confirming the unexpected results. However, as for this P0 group, there were no significant results when comparing survival between infected and non-infected individuals, the pathogen was not the cause for the quick death and probably age was at play. Infection with *Crithidia* could not be evaluated for this P0 group, as none of the bumblebees were infected.

- The presence of pathogens did result in a significant difference between some survival curves. Both *Apicystis* and *Vairimorpha* with p-values **0.0248** and **0.0284** respectively, had an effect on the survival of the P0 group during the first pesticide experiment. As the bumblebees of group P2 and P1 died very fast, this is linked to the high pesticide concentration and not to the place or presence of pathogens.

As seen in ***Figure 4.14***, the bumblebees infected with *Vairimorpha* died fast indicating that this pathogen reduces the lifespan of bumblebees. However, as this is the only significant result where *Vairimorpha* affects life span, further research is needed to gather more evidence.

The survival curves for bumblebees infected with *Apicystis* are odd, as only the non-infected agricultural bumblebees died fast and infected individuals lived longer. This result is counterintuitive and not logical when looking at the known effects of the pathogen. An explanation might be that these individuals carried the other two pathogens that were examined, but this was not the case. Remarkable is however that all individuals from this group (non-infected agricultural individuals) are from location L1, which perhaps indicates a location specific effect or the influence of age. A limit here is the small number of individuals in this specific group.

- The effect from pathogens was also determined by comparing different models with ANOVA. All p-values, χ^2 and df are shown in ***Appendix 3 (Table A3.9)***. Significant p-values **0.006195** and **0.04674** were again obtained for *Apicystis* and *Vairimorpha* for P0 group of pesticide experiment one, indicating the effect of the pathogen on survival time. One might conclude that *Vairimorpha* reduces survival time and that the odd result of *Apicystis* is because only the non-infected L bumblebees died fast, containing only L1 and having a low sample size. As the significant effect of pathogens is only seen once, we assume this did not interfere with the results of our original experiments.

- The presence of *Crithidia* in the S0 group of the nutrition experiment had a significant p-value **0.0376** using the Kaplan-Meier curves. However, the p-value was not significant when GLMM models were compared, indicating that *Crithidia* did not have an effect on survival time.

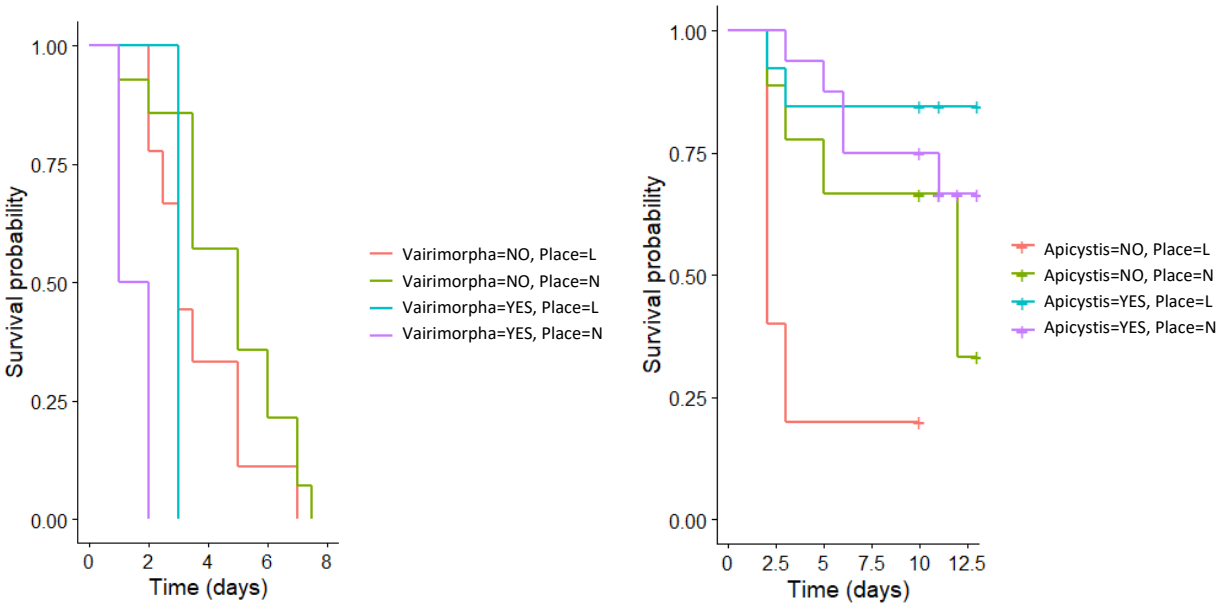

Figure 4.14: *Kaplan-Meier curves of bumblebees from place L/N and with/without the specific pathogen. During the first pesticide experiment, the P0 (0 µg/mL) group bumblebees died fast when* Nosema (Vairimorpha) *was present (Left), whereas the presence of* Crithidia *showed an unexpected result where non-infected agricultural individuals died fast (Right), which might be linked to a location specific effect or a low sample size.*

In general, we assume that our interpretation of the pesticide and nutrition experiments stay valid and that pathogens did not influence our results.

4.6.2.3 Influence of pathogens on the temperature experiment

For the temperature experiment, we calculated the mean critical temperature values from bumblebees, splitting the data into non-infected ("NO" group, none of the pathogens present) and infected ("YES" group, at least one pathogen present). The critical temperatures are slightly different, where infected ones seem less resistant to cold stress, having smaller boundaries as seen in ***Table 4.8***.

Table 4.8: *Critical temperatures for infected and non-infected* B. pascuorum.

	NOT INFECTED	INFECTED
CT_{min} (°C)	0.3	0.8
CT_{max} (°C)	47.8	47.6

In ***Table 4.9*** the individuals are compared, looking at the place and presence of pathogens. Again it is observed that the infected bumblebees have somewhat less capacity to regulate temperature having smaller boundaries, with a higher CT_{min} and a lower CT_{max}. The CT_{min} is almost the same for the agricultural non/infected bumblebees, whereas for the natural bumblebees there is a remarkable difference (-0.1°C and 0.6°C). When looking at the critical temperature differences between L and N bumblebees of healthy specimens, which was our initial goal, the CT_{min} (1.0°C and -0.1°C) differences are a lot bigger compared to when analysing all bumblebees together.

Based on this data, both pathogens and place could make a difference in temperature limits. It seems that natural bumblebees are better resistant to cold environments, whereas agricultural bumblebees are better at handling warmer environments. Infected individuals are less prone to survive more extreme temperatures.

One may not forget that these estimates contain values from bumblebees which all have different sizes. So further statistical analysis is necessary.

Table 4.9: *Critical temperatures for infected and non-infected* B. pascuorum *from L and N areas.*

	L		N	
	NOT INFECTED	INFECTED	NOT INFECTED	INFECTED
CT_{min} (°C)	1.0	1.1	-0.1	0.6
CT_{max} (°C)	48.0	47.8	47.7	47.5

Different models were compared using ANOVA, to see if the presence of the pathogens influences the converted critical temperature. This converted critical temperature value corresponds to the CT divided by the mean of the marginal cell lengths, to take the different size of the bumblebees into account. The results can be seen in ***Appendix 3*** **(*Table A3.10)***. For both CT_{min} and CT_{max}, all comparison models resulted in non-significant p-values, meaning that pathogens did not interfere with our temperature experiment and that the mean critical temperatures for all our bumblebees are similar.

Even though we saw variations in our calculated averages, the statistical values were not significant, indicating that there was no significant difference between CT for pathogens and place. This means that pathogens did not influence the capacity to regulate heat stress in bumblebees and that there were no significant CT differences between N and L bumblebees. However, it might be interesting to continue this study on more individuals.

5. DISCUSSION

5.1 PRELIMINARY REMARKS

For this thesis, the interest lies in the real life situation of bumblebees collected at different locations. Age and other interfering factors, such as intra individual variability (highly studied in humans, but also present in animals), might influence the outcome and interpretation of the results. To minimise these biases and variation and to increase power, the experiments were performed with a sufficiently large number of bumblebees, including control groups for each experiment.

To uncover possible stress-related adaptations between bumblebees from agricultural *versus* natural areas, the location aspect was taken into account. Bumblebees were collected from more than one location per habitat, more precisely from six locations: three agricultural and three natural areas, in order to avoid a location-specific aspect.

When bumblebees are reared under lab conditions or bought from commercial bumblebee producers, the exact age is known. In our case however, it was not possible to know the exact age of each individual, so age variations between bumblebees and different life stages of the colonies had to be taken into account.

5.2 PESTICIDES

During our pesticide experiments, we focussed on the **lethal effect** of Spinosad on bumblebees, by monitoring their survival time. The pesticide experiment was a short term research, lasting from a few days up to around three weeks, testing individual bumblebees. We opted for short term as before the start of the experiment, a part of the specimens from the first location had already died, which indicated uncertainties about the specimens' age or other possible interfering factors.

During the pesticide experiments, multiple concentrations of Spinosad were tested i.e. 0.04 µg/mL, 0.4 µg/mL, 4 µg/mL and 40 µg/mL.

The three highest concentrations (P2 = 40 µg/mL, P1 = 4 µg/mL and P4 = 0.4 µg/mL) had a lethal effect on bumblebees. The mean survival times were one day, two days and three days respectively, indicating that higher Spinosad concentrations are linked to a quicker death compared to lower concentrations. The lowest concentration (P3 = 0.04 µg/mL) did not have a lethal effect.

Our results are mostly in line with literature which also studied the effects of Spinosad. However, Abdu-Allah et al. (2011) stated that a 0.4 µg/mL Spinosad concentration was not harmful for *B. terrestris*. This might be linked to the different bumblebee species used.

The 4 µg/mL concentration showed a significant difference between the L and N bumblebees, whereby the individuals from agricultural areas seemed to live longer compared to these from natural areas. This confirms our **first hypothesis** which assumed that bumblebees living in agricultural areas, would survive longer with higher concentrations of pesticides compared to those in natural areas, since the former are more likely to have come into contact with pesticides previously, building up a tolerance. Future research might test concentrations closer to the value of 4 µg/mL, e.g. testing concentrations between 0.2 µg/mL and 20 µg/mL, to see if the same effects can be distinguished between agricultural

and natural bumblebees. Furthermore, it would be interesting to conduct more research comparing different bumblebee species and their resistance to various pesticide concentrations.

If the reverse would have been seen (N living longer than L), one could assume as an alternative that bumblebees living in agricultural areas had come into contact with pesticides before the experiment and crossed the threshold faster, resulting in a quicker death.

A possible expansion of our experiments would be to study the **sublethal effects** of low pesticide concentrations, such as the oviposition, emergence, amount of drones and reproducibility. To do this, bumblebees can be placed together in groups of six (female) workers, forming a microcolony with one female becoming dominant and producing unfertilised eggs. This set-up would be a long term experiment, lasting approximately two months. A similar set-up was already performed to evaluate sublethal effects in *B. terrestris*, with hives that were purchased from the company Biobest (Abdu-Allah et al., 2011). Similar experiments might be executed with field-caught bumblebees.

5.3 NUTRITION

No significant difference in mortality rate was observed for L and N bumblebees being fed different sugar concentrations of 25% and 50%. As there is no difference in survival capacity and all bumblebees can handle both sugar concentrations, these findings are not in line with our **second hypothesis**, which stated that bumblebees living in areas with limited food resources (agricultural areas) would survive longer with low food concentrations compared to bumblebees that are used to high food availability (natural areas). Further research might be conducted with more individuals, making it possible to include multiple lower sugar concentrations between 5% and 20%. A study by Piot (2020) also tested sugar concentrations of 50% and 25%, observing no significant differences in survival due to malnutrition. Piot (2020) indicated that the induced food stress might have been too limited to see an effect.

The volume (mL) intake per day per bumblebee was significantly different between the normal and reduced sugar concentrations. This indicates that bumblebees try to get a sufficient intake of sugar, resulting in more/less drinking depending on the concentration. The volume per day can be estimated at around 0.3 mL and 0.4 mL, for 50% and 25% sugar concentration respectively.

As there was no significant difference in the survival time of bumblebees with different sugar concentrations, we can assume that bumblebees (both L and N) are able to handle and survive the reduced concentration without any problems, but they have to compensate by drinking more of the food solution.

Nardone et al. (2013) also studied various sugar concentrations, solution types and intake volumes in *B. impatiens*. They concluded that, when bumblebees could choose in an artificially created foraging environment, higher sugar concentrations were preferred over low ones. Moreover, they suggest that sugar concentration might be more important than the intake volume. This last statement is in line with our observations of the intake volume.

5.4 TEMPERATURE

The mean critical temperatures for *B. pascuorum* correspond to CT_{min} ≈ 0.6 °C and CT_{max} ≈ 47.7 °C. For some videos in our experiment, the cameras did not record the whole session and some data was lost. However, since enough data was still available, both minimal and maximal critical temperatures could be obtained.

As mentioned before, research from Maebe et al. (2021a) on *B. terrestris* resulted in a CT_{min} ≈ -4.5°C and a CT_{max} ≈ 50°C. Research from Oyen and Dillon (2018) concluded that *B. impatiens* had a CT_{min} ≈ 4°C, ranging from 1.4°C to 8°C and CT_{max} ≈ 53°C, ranging from 42°C to 65°C. Our values do not deviate extremely from these, which indicates that our experiment is a good estimate for *B. pascuorum*.

The results are in line with our **third hypothesis**, that there is no significant difference between the critical temperatures of the L and N bumblebees. We did not expect to find a difference since the climate is the same for all our sampling locations in Belgium.

For our experiment set-up, more data could be collected to minimise position effects, which are linked to the varying temperatures between the different positions inside the incubator, e.g. difference between the sides and the middle positions in the incubator.

Future experiments may continue to discover the critical temperatures of other bumblebee species. Research is already ongoing, comparing bumblebee species living in different countries with more variations in climate. Moreover, one might also include to study the effect on males and females separately.

5.5 MORPHOLOGY

Literature states that the mass and relative body size of *B. pascuorum* (which is proportional to the length of the radial wing cell) is larger for drones compared to workers. This is confirmed in our results for drones and workers, where the average mass was 125.49 mg and 102.71 mg and average wing size was 3.10 mm and 2.88 mm, respectively. Moreover, when comparing left and right marginal wing cell sizes, there was no immediate indication for wing asymmetry.

The body size of *B. pascuorum* is estimated at 11 mm for drones, ten mm (small ones only seven mm) for workers and 13 mm for queens by Falk (2015), whereas Wilson-Rich (2016) estimated the body size of *B. pascuorum* between 12-14 mm for drones, 9-15 mm for workers and 15-18 mm for queens. Apart from body size estimation, no scientific research articles were found to compare our *B. pascuorum* measurements (mass, radial wing cells and the six leg compartments) with.

Our morphology measurements did not show remarkable differences between agricultural and natural bumblebees. However, previous studies from Eggenberger et al. (2019) on *B. pascuorum,* showed that the average body size of urban bumblebees was lower than that of rural populations. These results did not follow their expectations. They expected larger individuals in cities, as the fragmented distribution of floral resources might need larger foraging distance and capacity. Moreover, clear differences (e.g. body size, proboscis length) were noticed between urban and rural bumblebees of the same species in this research, making additional research interesting (Eggenberger et al., 2019). Therefore, also this topic deserves more attention.

Our data will be openly available and can be consulted by external parties. In ***Appendix 5***, a link to the morphology sheets is included. This might be the beginning of a database with shared information about detailed mass, radial wing cell sizes, length of the six leg compartments and morphology sheets with both wings to study wing symmetry and other measurements of *B. pascuorum*. At a later stage, different bumblebee species from various countries can be included.

5.6 PATHOGENS

The incidence of pathogens in our evaluation is in line with the results in literature, mentioning an incidence of around 50% for *Apicystis*, 20% for *Crithidia* and 20% for *Vairimorpha* (Vanderplanck et al., 2019; Ocepek et al., 2021). A study by Piot (2020) also found a prevalence of 48.8% for *Apicystis*, 33.6% for *Crithidia* and 15.2% for *Vairimorpha*. Our positive groups show similar results, i.e. 40.4%, 20.8% and 14.0% respectively.

In literature, 50% of bumblebees are estimated to be infected with one pathogen and only 10% with multiple pathogens (Vanderplanck et al., 2019; Ocepek et al., 2021). Our evaluation resulted in 43.6% having one and 17.6% having multiple pathogens.

All pathogens were present at all locations and place did not show a significant difference. However, our results showed that the time of collection (batch) influenced the presence of pathogens, with lower levels of infection at the end of September. More research might be worthwhile to study the presence of pathogens at more geographical locations and over a broader time frame.

The conopid fly parasite was only found in bumblebees that died before they could be used in the experiments. Therefore, it is safe to say that the presence of this parasite did not interfere with our other results. The presence of the conopid fly in 12.1% of the cases was unexpected, as we did not intend to screen or look for this parasite, but it caused an early death for part of the bumblebees collected, proving the severe and lethal effect, as was also described by Mundy et al. (2011).

As research determining differences in incidence and influence of pathogens between bumblebees from agricultural and natural areas is scarce or non-existent, research should be continued to uncover more about possible interactions.

Research performed by Piot (2020) with *A. bombi* in *B. terrestris*, noted there was a significant difference in survival for infected and non-infected individuals, where faster mortality was observed for diseased bumblebees. This indicates that the presence of pathogens did influence the survival time of the bumblebee. In our research however, we were not able to conclude that *Apicystis* had a negative impact on survival.

Almost no differences were found in survival capacity between infected and non-infected individuals. The pathogens did not influence the experiments and did not alter the survival capacity or critical temperatures. The pathogens might not have had an effect yet or needed more time before the symptoms could arise and bumblebees became weaker e.g. dormant spores for *Vairimorpha*. Another explanation could be that the pathogens were only present in very small concentrations which did not lead to severe diseases, or that bumblebees were tolerant, e.g. the pathogens did not have a big effect on the host's fitness or the host could resist the pathogens by controlling harmful effects caused by the infection (Piot, 2020). As both infected and non-infected bumblebees died at various times and almost all results are non-significant, the pathogens did not seem to play a crucial role in our experiments, making the outcomes more trustworthy.

5.7 GENES RELATED TO ADAPTATIONS TO STRESS FACTORS

In future research, one might perform DNA/RNA extractions targeting candidate genes, to discover if they are linked to adaptations and possible mutations explaining the enhanced tolerance and change of function and behaviour of bumblebees in climates with changing stress factors. Even though essential research to establish these candidate genes is ongoing (Hart et al., 2022), there is at this moment too much uncertainty. In the future, one might be able to link the visual, behavioural and morphological differences and adaptations between bumblebees in natural and agricultural areas to the correct genes and their corresponding functions.

6. CONCLUSIONS

During the **pesticide experiment**, high Spinosad concentrations of 40 µg/mL, 4 µg/mL and 0.4 µg/mL were lethal for bumblebees, whereas a low Spinosad concentration of 0.04 µg/mL seemed harmless. The intermediate concentration P1 corresponding to 4 µg/mL showed interesting results with a significant difference between agricultural and natural bumblebees. Agricultural bumblebees survived longer and might be better adapted to the pesticides as they already built up a resistance by previous contact with pesticides.

For the **nutrition experiment**, the reduced sugar concentration (25%) did not have an impact on the bumblebees' survival. No differences between agricultural and natural bumblebees were observed. The nutritional stress which was induced might have been too limited as the bumblebees could handle both concentrations.

It was clear that the volume consumed by bumblebees receiving a lower sugar water concentration (25%) was significantly bigger than that being consumed by bumblebees receiving a normal sugar water concentration (50%). This indicates that the total sugar intake was compensated by drinking more of a solution which contained less sugar.

During the **temperature experiment**, no differences were observed between the critical temperature of agricultural and natural bumblebees. As the specimens were collected on a relatively small geographical scale in Belgium with a similar climate on all locations, they did not have to adapt to other temperatures. The mean CT_{min} and CT_{max} for *B. pascuorum* resulted in 0.6°C and 47.7°C, respectively.

The **morphology** and **pathogens** evaluation make the interpretation of the results more complex. As size influences a bumblebees' capacity to regulate heat and as pathogens can decrease its survival chances in a stressful environment, these interfering factors were taken into account.

Apicystis, *Crithidia*, *Vairimorpha* and the conopid fly had an incidence of 40.4%, 20.8%, 14.0% and 12.1% respectively. The conopid fly caused a quick death in bumblebees even before these could be used in the experiments, which indicates the danger of this parasite. The other pathogens *Apicystis*, *Crithidia* and *Vairimorpha* did not cause increased mortality and did not influence the outcome of the original experiments.

In the future, similar experiments can be performed expanding to various bumblebee species and testing individuals on a broader geographical scale and continuing to uncover differences between bumblebees from agricultural and natural areas. Pesticide experiments could be performed with Spinosad concentrations from 0.2 µg/mL to 20 µg/mL, also studying sublethal effects and nutrition experiments with increased nutritional stress only containing 5% to 20% sugar. Testing pathogen interference and influence in the same experiments and bumblebee species could be done with bumblebees with a known age from a controlled environment.

Numerous experiments are possible to expand our knowledge and gain a better understanding of the whole bumblebee story with combinations of stress factors, which can result in adaptations. The path to uncover all the traits that increase the bumblebees' fitness will be long, but really worth it!

"Bee" sweet and "Bumble" to every creature in the world!

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8. APPENDICES

Appendix 1: *Bombus pascuorum* information sampling locations

Appendix 2: Excel and csv files with data from all experiments

Appendix 3: Additional values statistical evaluation: pesticide/nutrition experiments and influence of pathogens

Appendix 4: Basic script used in R Studio

Appendix 5: Morphology sheets *B. pascuorum*

Appendix 6: Presence of pathogens: number of bumblebees (incidence of different pathogens (#) in bumblebees from different locations, collected in batches on various dates)

Appendix 1

Bombus pascuorum information sampling locations

Location 1: Zwalm / Zottegem	**L1:** agricultural area 1	# = **150** bumblebees
Sampling dates	1st batch 04/08/2022 2nd batch 13/08/2022 3rd batch 21/08/2022	
Coordinates	50.867299 , 3.767169 (A-B-C-D-E-F-G-H-I)	
Flowers	*Galeopsis tetrahit* *Cirsium palustre* *Nepeta racemosa* *Epilobium hirsutum*	
Remark	No bumblebees were found at the end of September, this might be linked to the colony age or limited food resources.	

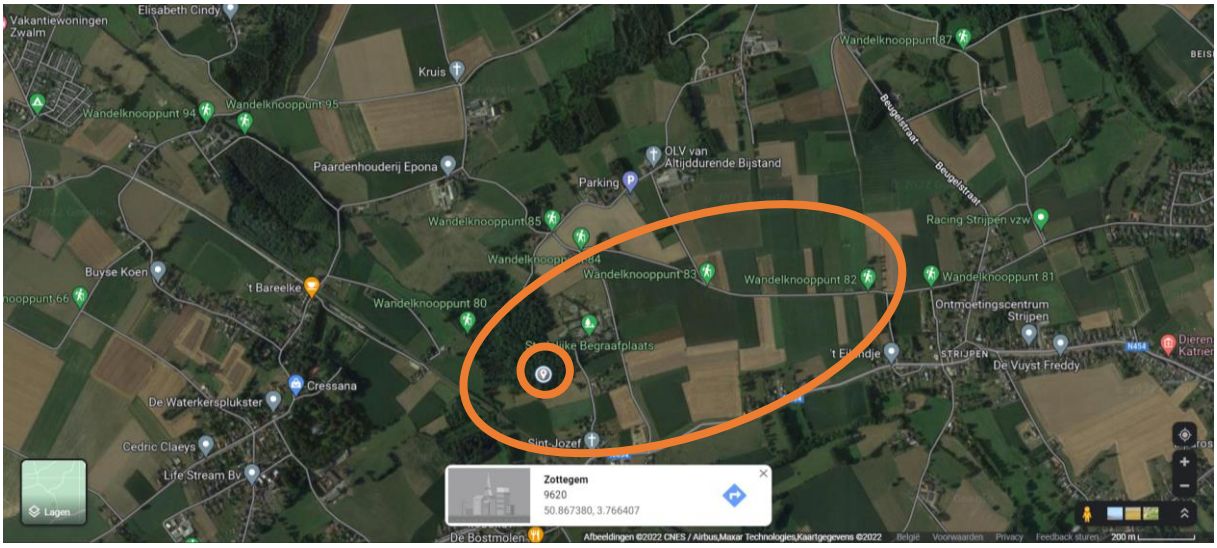


Figure A1.1: *Location 1: Zwalm - Zottegem. Agricultural area "L1".*

Location 2: Beervelde (Vogelzangstraat, Vispoelstraat)	**L2:** agricultural area 2	# = **127** bumblebees
Sampling dates	1st batch 05/08/2022 2nd batch 21/08/2022 3rd batch 21/09/2022	
Coordinates	51.076794 , 3.94452 (A) 51.076799 , 3.942557 (B) 51.079997 , 3.939238 (C-D-E-F)	
Flowers	*Cirsium arvense* *Calystegia sepium* *Impatiens glandulifera*	
Remark	Also a lot of *B. lapidarius* and *B. terrestris* present at 1st and 2nd coordinates.	

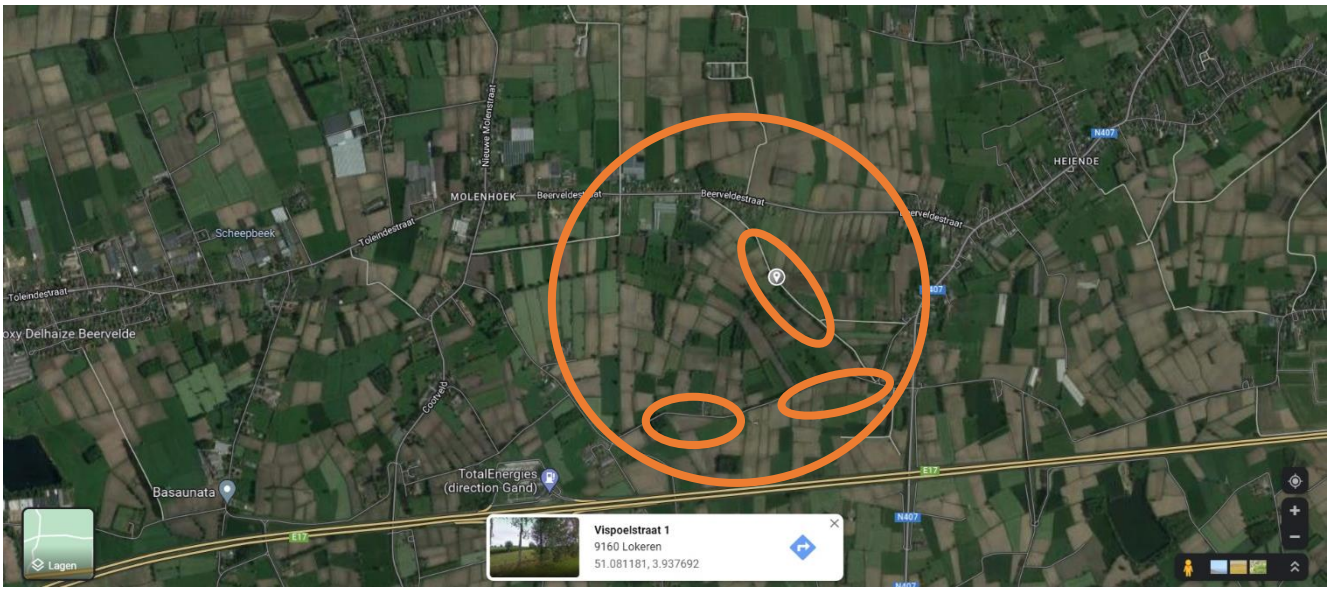


Figure A1.2: *Location 2: Beervelde (Vogelzangstraat, Vispoelstraat). Agricultural area "L2".*

Location 3: Poeke / Vinkt (between Tielt and Aalter)	**L3:** agricultural area 3	# = **135** bumblebees
Sampling dates	1st batch 09/08/2022 2nd batch 21/08/2022 3rd batch 21/09/2022	
Coordinates	51.029177 , 3.521163 (A) 51.025342 , 3.518263 (B) 51.032358 , 3.449301 (C-D-E-F-G)	
Flowers	*Trifolium pratense* *Cirsium arvense* *Cichorium intybus* *Epilobium hirsutum*	
Remark	A lot of big bumblebees present.	

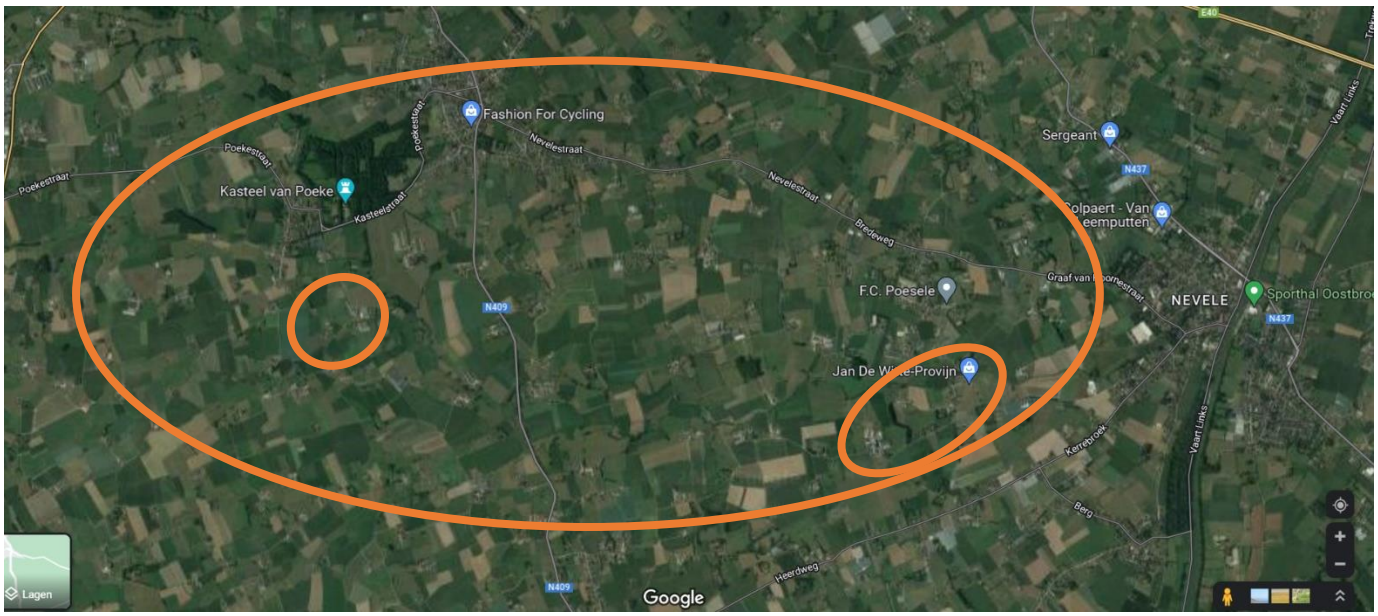


Figure A1.3: *Location 3: Vinkt / Poeke (between Tielt and Aalter). Agricultural area "L3".*

Location 4: Buggenhout (Buggenhoutbos)	**N1:** natural area 1	# = **97** bumblebees
Sampling dates	1st batch 07/08/2022 2nd batch 21/08/2022	
Coordinates	50.998850 , 4.222746 (A) 51.002825 , 4.227879 (B-C) 50.995472 , 4.214829 (E) 51.002587 , 4.229468 (D-F)	
Flowers	*Lythrum salicaria* *Teucrium scorodonia* *Symphytum officinale* *Cirsium arvense* *Epilobium parviflorum*	
Remark	Heath was located inside the forest. Also the European hornet species (*Vespa crabro*) was present. No bumblebees were found at the end of September, this might be linked to the colony age or limited food resources. Moreover, part of the area was now used as grazing pasture for goats.	

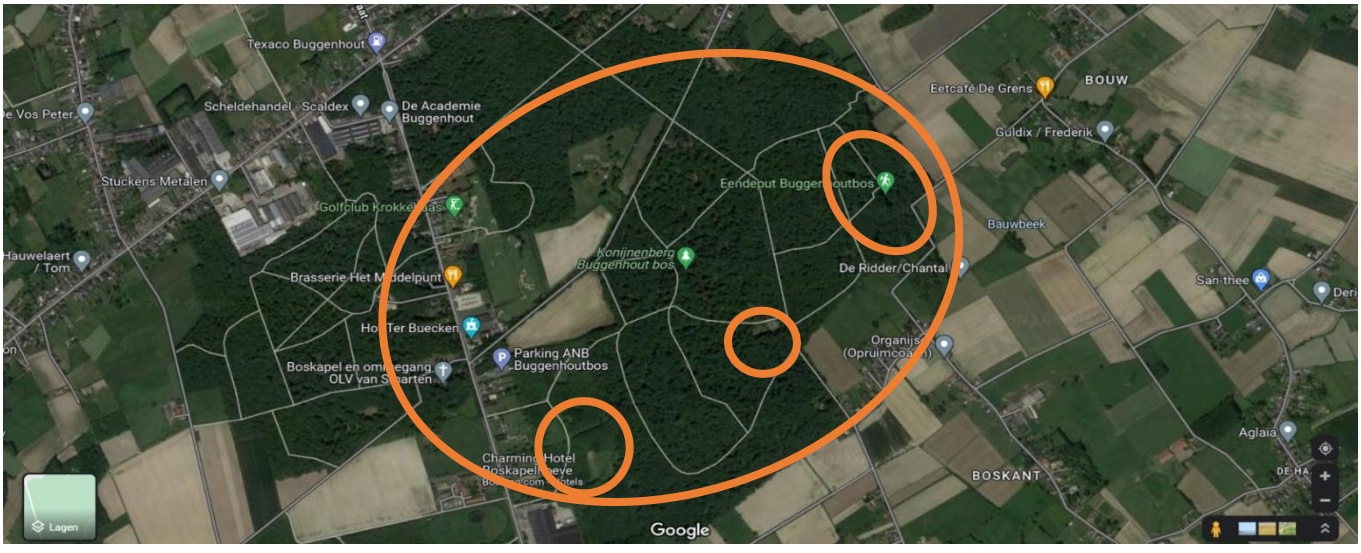


Figure A1.4: *Location 4: Buggenhout (Buggenhoutbos). Natural area 1 "N1".*

Location 5: Dendervallei Ninove / Zandbergen	**N2:** natural area 2	# = **133** bumblebees
Sampling dates	1st batch 08/08/2022 2nd batch 23/08/2022 3rd batch 22/09/2022	
Coordinates	50.809831 , 3.979160 (A-B-C) 50.808728 , 3.979360 (D-E-F)	
Flowers	*Trifolium pratense* *Impatiens glandulifera* *Cirsium arvense* *Calystegia sepium* *Vicia cracca*	
Remark	Larger bumblebees on the *Impatiens glandulifera* compared to bumblebees on the other flowers.	

Figure A1.5: *Location 5: Dendervallei Ninove - Zandbergen. Natural area 2 "N2".*

Location 6: Kluisbergen (Kluisbos)	**N3:** natural area 3	# = **96** bumblebees
Sampling dates	1st batch 10/08/2022 2nd batch 21/08/2022 3rd batch 21/09/2022	
Coordinates	50.759224 , 3.489160 (A) 50.759553 , 3.496766 (B-C-D-E-F)	
Flowers	*Arctium tomentosum* *Calluna vulgaris*	
Remark	A lot of small bumblebees present. Heath was located inside the forest. No *Bombus pascuorum* found at other natural area (visited on 10/08/2022) around "Waregem Spitaalbossen": (Waregemseweg, Zilverberg, Tjammelstraat).	

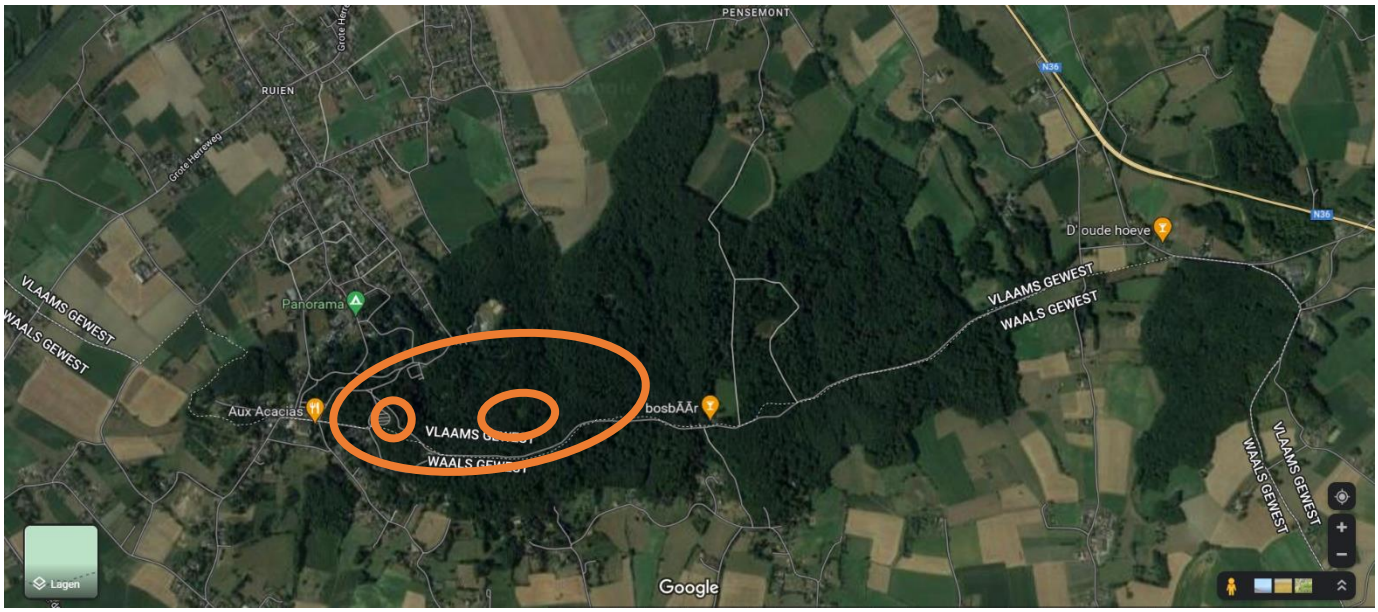


Figure A1.6: *Location 6: Kluisbergen (Kluisbos). Natural area 3 "N3".*

Appendix 2

Excel and csv files with data from all experiments

All data can be consulted via the following links:

1. Pesticide and nutrition experiments (survival) Excel

1. Pesticide and nutrition experiments (survival) csv file

2. Nutrition mL consumed Excel

2. Nutrition mL consumed csv file

3. Temperature experiment (critical temperature) Excel

3. Temperature experiment (critical temperature) csv file

4. Morphology (measurements) Excel

4. Morphology (measurements) csv file

5. Pathogen detection (*Apicystis*, *Crithidia*, Vairimorpha and conopid fly) Excel

5. Pathogen detection (*Apicystis*, *Crithidia*, Vairimorpha and conopid fly) csv file

6. Temperature and pathogens Excel

6. Temperature and pathogens csv file

Appendix 3

Additional values statistical evaluation:

pesticide/nutrition experiments and influence of pathogens

Pesticide experiment one

Spinosad concentration:

- P2 = 40 µg/mL
- P1 = 4 µg/mL
- P0 = 0 µg/mL

Table A3.1: *pesticide experiment one: chi-squared (χ^2), degree of freedom (df) and p-values. Is there a significant difference in survival for the different concentrations?*

		χ^2	Df	p-value
All N	P2 *versus* P1	8.3	1	0.004
	P2 *versus* P0	62.8	1	2e-15
	P1 *versus* P0	57.6	1	3e-14
All L	P2 *versus* P1	19.4	1	1e-05
	P2 *versus* P0	45.3	1	2e-11
	P1 *versus* P0	34.2	1	5e-09

Table A3.2: *pesticide experiment one: chi-squared (χ^2), degree of freedom (df) and p-values. Is there a significant difference in survival for the L and N bumblebees?*

	χ^2	Df	p-value
P2: N *versus* L	0	1	0.88
P1: N *versus* L	5	1	**0.025**
P0: N *versus* L	0.6	1	0.43

Pesticide experiment two

Spinosad concentration:

- P4 = 0.4 µg/mL
- P3 = 0.04 µg/mL
- P0 = 0 µg/mL

Table A3.3: *pesticide experiment two: chi-squared (χ^2), degree of freedom (df) and p-values. Is there a significant difference in survival for the different concentrations?*

		χ^2	df	p-value
All N	P4 *versus* P3	3.4	1	0.063
	P4 *versus* P0	8.6	1	0.0033
	P3 *versus* P0	1	1	0.32
All L	P4 *versus* P3	1.6	1	0.2
	P4 *versus* P0	1.4	1	0.24
	P3 *versus* P0	0.1	1	0.76

Table A3.4: *pesticide experiment two: chi-squared (χ^2), degree of freedom (df) and p-values. Is there a significant difference in survival for the L and N bumblebees?*

	χ^2	Df	p-value
P4: N *versus* L	0.1	1	0.7
P3: N *versus* L	0.4	1	0.51
P0: N *versus* L	5.5	1	**0.019**

Table A3.5: *pesticide experiment two: chi-squared (χ^2), degree of freedom (df) and p-values. Is there a significant difference in survival when comparing all N with L2&L3 (**excluding L1**) bumblebees.*

	χ^2	Df	p-value
P4: N *versus* L	0.1	1	0.72
P3: N *versus* L	0	1	0.92
P0: N *versus* L	3	1	0.082

Nutrition experiment

Sugar water concentration:

- S1 = 25%
- S0 = 50%

Table A3.6: *nutrition experiment: chi-squared (χ^2), degree of freedom (df) and p-values. Is there a significant difference in survival for the different concentrations?*

	χ^2	Df	p-value
All N: S1 *versus* S0	0	1	0.83
All L: S1 *versus* S0	3.8	1	0.052

Table A3.7: *nutrition experiment: chi-squared (χ^2), degree of freedom (df) and p-values. Is there a significant difference in survival for the L and N bumblebees?*

	χ^2	Df	p-value
S1: N *versus* L	1.8	1	0.18
S0: N *versus* L	0	1	0.92

Influence of pathogens on survival and critical temperatures

Table A3.8: *Effect of each pathogen on survival time by plotting Kaplan-Meier curves and performing coxph. The reference is "not infected No" and "place L". Bumblebees with YES and NO groups are compared for the three pathogens for every experiment and corresponding treatment group. The p-value was calculated for each variable: pathogen and place.*

Pathogen	Experiment	Concentration	Predictor variable	p-value
Apicystis	Pesticide exp 1	P2	*Apicystis* = Yes	0.0648
			Place = N	0.3939
		P1	Yes	0.629
			N	**0.033**
		P0	Yes	**0.0248**
			N	0.3734
	Pesticide exp 2	P4	Yes	0.603
			N	0.218
		P3	Yes	0.608
			N	0.517
		P0	Yes	0.1817
			N	**0.0232**
	Nutrition exp	S1	Yes	0.843
			N	0.273
		S0	Yes	0.870
			N	0.476
Crithidia	Pesticide exp 1	P2	*Crithidia* = Yes	0.195
			Place = N	0.648
		P1	Yes	0.1241
			N	**0.0298**
		P0	Yes	0.326
			N	0.624
	Pesticide exp 2	P4	Yes	0.271
			N	0.315
		P3	Yes	0.365
			N	0.337
		P0	Yes	/
			N	**0.019**
	Nutrition exp	S1	Yes	0.490
			N	0.161
		S0	Yes	**0.0376**
			N	0.1711
Vairimorpha	Pesticide exp 1	P2	*Vairimorpha* = Yes	0.740
			Place = N	0.673
		P1	Yes	0.671
			N	0.435
		P0	Yes	**0.0284**
			N	0.3843
	Pesticide exp 2	P4	Yes	0.0572
			N	0.2015
		P3	Yes	0.243
			N	0.628

		P0	Yes	0.9026
			N	**0.0263**
	Nutrition exp	S1	Yes	0.0825
			N	0.1174
		S0	Yes	0.194
			N	0.735

Table A3.9: *Effect of each pathogen on survival time by comparing models with ANOVA, resulting in chi-squared (χ^2), degree of freedom (df) and p-values. Bumblebees with YES and NO groups are compared for the three pathogens for every experiment and corresponding treatment.*

Pathogen	Experiment	Concentration	χ^2	df	p-value
Apicystis	Pesticide exp 1	P2	4.6565	2	0.09746
		P1	1.0646	2	0.5873
		P0	10.168	2	**0.006195**
	Pesticide exp 2	P4	0.2094	2	0.9006
		P3	3.7694	2	0.1519
		P0	3.3816	3	0.1844
	Nutrition exp	S1	5.1625	2	0.07568
		S0	0.6644	2	0.7174
Crithidia	Pesticide exp 1	P2	2.1919	2	0.3342
		P1	4.5946	2	0.1005
		P0	2.0565	2	0.3576
	Pesticide exp 2	P4	1.8456	2	0.3974
		P3	0.8557	1	0.355
		P0	/	/	/
	Nutrition exp	S1	1.8037	2	0.4058
		S0	4.792	2	0.09108
Vairimorpha	Pesticide exp 1	P2	0.1169	2	0.9432
		P1	0.1589	1	0.6902
		P0	6.1264	2	**0.04674**
	Pesticide exp 2	P4	4.7399	2	0.09349
		P3	0.4609	1	0.4972
		P0	0.0498	1	0.8234
	Nutrition exp	S1	2.8282	1	0.09262
		S0	3.5718	2	0.1676

Table A3.10: *Effect of each pathogen on critical temperatures by comparing models with ANOVA, resulting in chi-squared (χ^2), degree of freedom (df) and p-values. Infected individuals contain at least one YES group and are compared with non-infected individuals which only contain NO groups. Bumblebees with YES and NO groups are compared for the other three pathogens.*

		χ^2	Df	p-value
Infected	CT_{min}	0.3425	2	0.7426
	CT_{max}	1.8312	2	0.4003
Apicystis	CT_{min}	0.3052	2	0.8585
	CT_{max}	3.9444	2	0.1392
Crithidia	CT_{min}	0.9466	2	0.623
	CT_{max}	1.2449	2	0.5366
Vairimorpha	CT_{min}	1.9887	2	0.37
	CT_{max}	0.5746	2	0.7503

Appendix 4

Basic script used in R Studio

Textbox A4.1: *R code to evaluate temperature experiment with generalised linear models.*

```
### Code in R Studio
# temp is the dataset containing all the information of this experiment.

# calculating a value to compare bumblebees from different locations, taking into account size (related to radial wing cell) differences.
extra_value_cold <- temp$T_env_mean_cold/temp$Wing_mean
extra_value_hot <- temp$T_env_min_hot/temp$Wing_mean

Example1 <- glm(extra_value_cold ~ place, data = temp)        # L / N
Example2 <- glm(extra_value_cold ~ location, data = temp)     # Locations
Example3 <- glm(extra_value_hot ~ place, data = temp)         # L / N
Example4 <- glm(extra_value_hot ~ location, data = temp)      # Locations

# For the final results, information of the separate place and location were used as before, getting results for both the minimal and maximal critical temperature.
Summary(Example1)
Summary(Example2)
Summary(Example3)
Summary(Example4)
```

Textbox A4.2: *R code to evaluate morphology with different generalised linear models to determine if there is a significant difference for the "mass" of the bumblebees.*

```
### Code in R Studio
# morphology is the dataset containing all the information of this experiment.

Example1 <- glm(mass ~ M.V, data = morphology)                # Male / Female
Example2 <- glm(mass ~ place, data = m_worker)                # L / N
Example3 <- glm(mass ~ location, data = m_worker)             # Locations (L1,L2,L3,N1,N2,N3)
Example4 <- glm(mass ~ place+location, data = m_worker)       # place and location, NO interaction
Example5 <- glm(mass ~ place*location, data = m_worker)       # place and location, with interaction

# For the final results, information of different sex and of the separate place and location were used.
Summary(Example1)
Summary(Example2)
Summary(Example3)
```

Textbox A4.3: *R code to evaluate survival during pesticide and nutrition experiments with and without the influence of pathogens.*

```
### Code in R Studio
# pesticidedata is the dataset containing all the information.

library(ggplot2)
library(tidyverse)
library(dplyr)
library(tidyr)
library(survival)
install.packages("survminer")
library(survminer)

# Kaplan-Meier Survival curves with log-rank test
surv_object <- Surv(time = pesticidedata$Days.alive, event = pesticidedata$Ev == "1")
fit1 <- survfit(surv_object ~ place, data = pesticidedata)
summary(fit1)
ggsurvplot(fit1, data = pesticidedata, pval = TRUE, xlab = "Time (days)", legend = "right")
survdiff(surv_object ~ place, data= pesticidedata)

# Kaplan-Meier Survival curves with the cox proportional hazard model
surv_object <- Surv(time = survivaldata$Days.alive, event = survivaldata$Ev==1)
fit1 <- survfit(surv_object ~ pathogen + place, data = survivaldata)
summary(fit1)
ggsurvplot(fit1, data = survivaldata, pval = TRUE, xlab = "Time (days)", legend = "right")
coxfit <- coxph(Surv(survivaldata$Days.alive) ~ pathogen + place, data = survivaldata, ties = 'exact')
summary(coxfit)
```

***Textbox A4.4**: R code to evaluate if pathogens influenced the previous experiments.*

```
### Code in R Studio
# pathogendata and pathogen_tempdata are the datasets containing all the information.
install.packages("lme4")
library(lme4)

# 1: Do place or batch influence the presence of pathogens?
model <- glmer(pathogen ~ place + batch + (1 | location), data = pathogendata, family = "binomial")
summary(model, corr = FALSE)
model0 <- glmer(pathogen ~ batch + (1 | location), data = pathogendata, family = binomial)
summary(model, corr = FALSE)
anova(model,model0)
model02 <- glmer(pathogen ~ place + (1 | location), data = pathogendata, family = binomial)
summary(model, corr = FALSE)
anova(model,model02)

# 2: Do pathogens influence the survival time?
# per pathogen, per experiment and per treatment group
model <- lmer (Days.alive ~ pathogen * place + (1 | location), data = pathogendata)
summary(model, corr = FALSE)
model0 <- lmer (Days.alive ~ place + (1 | location), data = pathogendata)
summary(model, corr = FALSE)
anova(model,model0)

# 3: Do pathogens influence the critical temperatures?
# per pathogen and for both minimal and maximal critical temperature
model <- lmer (extra_value_hot ~ pathogen * place + (1 | location), data = pathogen_tempdata)
summary(model, corr = FALSE)
model0 <- lmer (extra_value_hot ~ place + (1 | location), data = pathogen_tempdata)
```

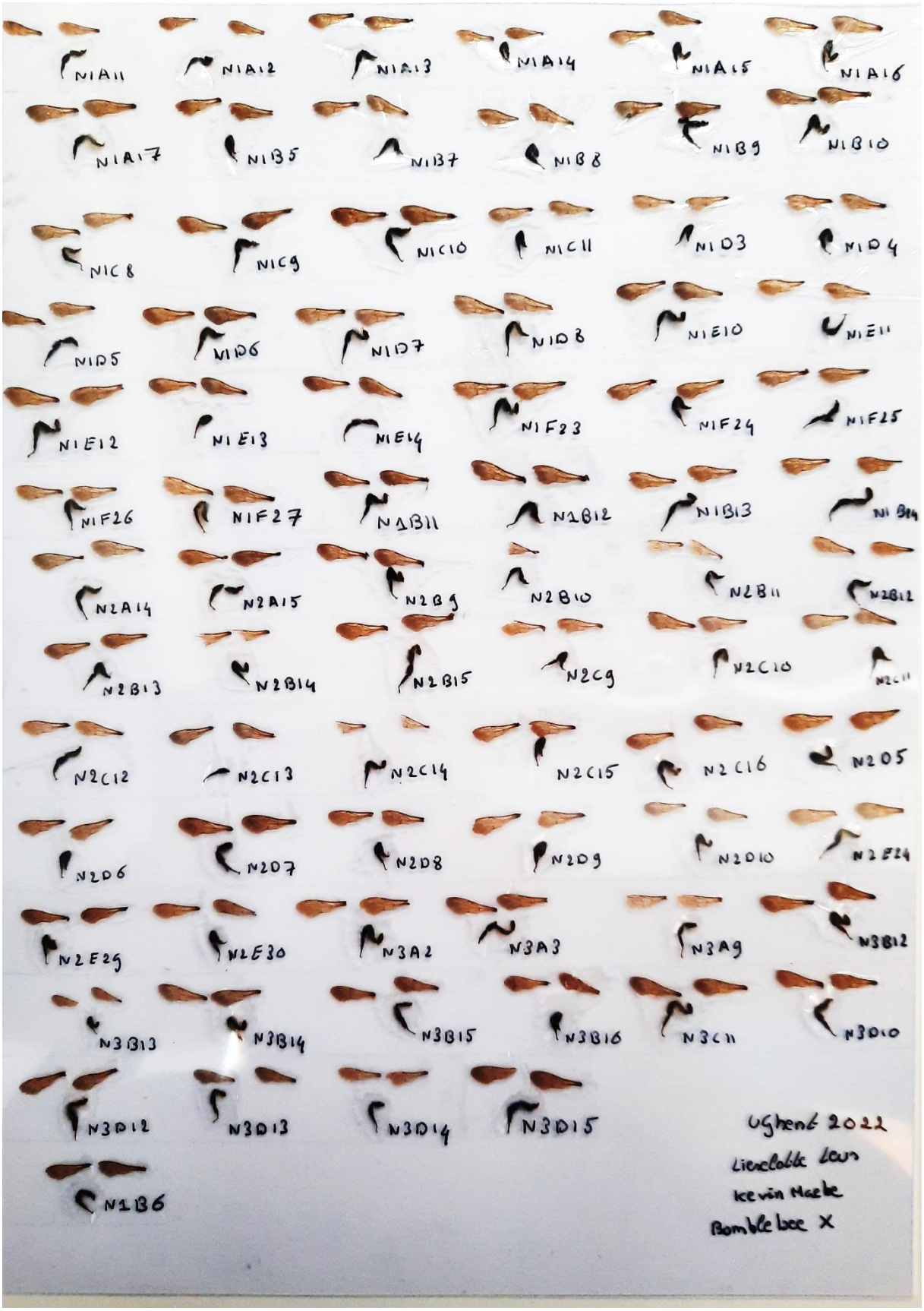
Appendix 5

Morphology sheets *B. pascuorum*

One example of a morphology sheet is shown below.

All 11 morphology sheets can be found via the following link:

Morphology sheets


N1A11
N1A12
N1A13
N1A14
N1A15
N1A16
N1A17
N1B5
N1B7
N1B8
N1B9
N1B10
N1C8
N1C9
N1C10
N1C11
N1D3
N1D4
N1D5
N1D6
N1D7
N1D8
N1E10
N1E11
N1E12
N1E13
N1E14
N1F23
N1F24
N1F25
N1F26
N1F27
N1B11
N2B12
N1B13
N2A14
N2A15
N2B9
N2B10
N2B11
N2B12
N2B13
N2B14
N2B15
N2C9
N2C10
N2C12
N2C13
N2C14
N2C15
N2C16
N2D5
N2D6
N2D7
N2D8
N2D9
N2D10
N2E24
N2E25
N2E30
N3A2
N3A3
N3A9
N3B12
N3B13
N3B14
N3B15
N3B16
N3C11
N3D10
N3D12
N3D13
N3D14
N3D15
N1B6
Ughent 2022
Lieveloble lous
kevin Haeke
Bomble bee X

Appendix 6

Presence of pathogens: number of bumblebees

Table A6.1: *Incidence of different pathogens (#) in bumblebees from different locations, collected in batches on various dates.*

LOCATION	BATCH	APICYSTIS #			CRITHIDIA #			VAIRIMORPHA #			CONOPID FLY #	
		Yes	Maybe	No	Yes	Maybe	No	Yes	Maybe	No	Yes	No
L1	1 (04/08)	48	2	23	6	9	58	7	4	62	24	49
	2 (13/08)	10	5	31	4	7	35	7	0	39	9	37
	3 (21/08)	7	3	19	0	3	26	1	3	25	1	28
L2	1 (05/08)	28	6	37	29	8	34	9	4	58	17	54
	2 (21/08)	8	2	17	4	5	18	7	6	14	1	26
	3 (21/09)	6	0	23	5	2	22	1	6	22	0	29
L3	1 (09/08)	27	9	39	17	4	54	16	2	57	9	66
	2 (21/08)	6	3	20	5	1	23	3	2	24	3	26
	3 (21/09)	9	6	16	4	7	20	1	2	28	0	31
N1	1 (07/08)	38	4	28	16	9	45	16	6	48	10	60
	2 (21/08)	14	1	12	4	1	22	1	3	23	24	30
N2	1 (08/08)	35	7	23	21	8	36	11	5	49	8	57
	2 (23/08)	15	4	11	0	1	29	1	2	27	2	28
	3 (22/09)	16	5	17	18	6	14	7	9	22	0	38
N3	1 (10/08)	21	13	25	13	1	45	10	7	42	2	57
	2 (21/08)	2	7	10	0	0	19	3	4	12	0	19
	3 (21/09)	7	2	9	7	4	7	2	2	14	0	18