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Influence of social and environmental context on bumblebee nutrition

Stéphane Kraus

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THÈSE

En vue de l'obtention du
DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE
Délivré par l'Université Toulouse 3 - Paul Sabatier

Présentée et soutenue par
Stéphane KRAUS

Le 15 juin 2022

**Influence du contexte social et environnemental sur la nutrition
des bourdons**

Ecole doctorale : **BSB - Biologie, Santé, Biotechnologies**

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Unité de recherche :

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**Influence of social and
environmental context on
bumblebee nutrition**

Most of the works were created by the author

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Abstract

For a few decades, the development of nutritional geometry has brought new insights into how individual animals eat and balance their acquisition of multiple nutrients simultaneously to maximize overall fitness. Yet, in social species, such as ants and bees, diet balancing is ensured by a minority of individuals that need to choose foods in order to meet their own needs as well as those of all other colony members whose needs may differ according to age, sex, caste, and the environmental conditions.

In this thesis, I used nutritional ecology to study how bumblebees *Bombus terrestris* balance their nutrient collection across social and ecological contexts. To do so I designed cafeteria experiments in which individual bees or micro-colonies could balance their diet from artificial diets varying in their composition of carbohydrates, proteins and lipids.

In the first chapter, I measured the response of individual bumblebees to each of the three nutrients in food and confirmed they respond more to carbohydrates than to lipids or proteins. Then, we let individuals' bees of various ages and castes time to self-compose their nutritional balance between varying diets that differ in their macronutrient's concentration. Nutrient regulation was no age- nor body size-dependant. Queen and workers non-randomly converged to different nutritional diets, essentially toward proteins or carbohydrates, respectively. Foragers in a third similar experiment, but outside in a flight tunnel, demonstrated a generalist collect by non-randomly visiting several artificial flowers to compose a specific nutritional diet.

In the second chapter, I studied the influence of brood on food collection by the workers. When constrained to single diets, micro-colonies without brood did not regulate proteins, resulting in their over-collection.

In the third chapter I examined the effect of environmental pressures on behaviour and fitness, by observing nutritional choices of bumblebees at various temperatures. At optimal temperature (30°C), bees self-composed a diet to maximize their reproductive ability; while at low temperature, they searched for carbohydrates to prioritize survival; and at high temperature, they searched for water and/or lipid in diets.

Bumblebees as pollinators have a noteworthy economic value since their domestication, and with the current widespread declines of wild bees, manipulating the nutritional behaviour of domesticated species could selectively increase foraging activity and mitigate the ongoing pollination crisis.

Résumé

Depuis plusieurs décennies maintenant, la géométrie nutritionnelle a permis d'observer comment divers animaux équilibrent leur apport en plusieurs nutriments de manière simultanée afin de maximiser leurs performances générales. Cependant, chez les espèces sociales, tel que les fourmis ou les abeilles, la régulation nutritionnelle est assurée par une minorité d'individus qui choisissent leur nourriture en fonction non seulement de leurs propres besoins, mais aussi de ceux des autres membres de la colonie. Les besoins des autres membres peuvent varier selon l'âge, le sexe, la caste ou encore les conditions environnementales.

Durant cette thèse, via l'approche de géométrie nutritionnelle, j'ai étudié comment les bourdons, *Bombus terrestris*, régulent leur prise de nutriments en fonction du contexte social et écologique. Pour cela j'ai mis au point des expériences dites de cafétéria, dans lesquelles des bourdons isolés ou en micro-colonies pouvaient réguler leurs régimes alimentaires à partir de plusieurs diètes artificielles, variées en concentrations de sucres, protéines et lipides.

Dans le premier chapitre, j'ai mesuré la réponse des bourdons à chacun de ces macronutriments et ainsi confirmé qu'ils sont plus motivés aux sucres plutôt qu'aux lipides ou protéines. Ensuite j'ai laissé le temps à des bourdons isolés, de divers âges et castes, de composer leur régime alimentaire à partir de plusieurs diètes artificielles. Leur régulation nutritionnelle n'était ni âge ni taille dépendante. Les reines et les ouvrières convergeaient vers divers régimes, respectivement plus dirigés vers les protéines ou vers les sucres. Les butineuses, étudiées dans une troisième expérience faite en extérieur dans un tunnel de vol, démontrèrent une collecte nutritionnelle généraliste en visitant de manière non-aléatoire divers fleurs artificielle afin de composer un régime alimentaire précis.

Dans le deuxième chapitre, j'ai étudié l'influence du couvain sur le comportement de collecte alimentaire des ouvrières. Quand contraintes à un régime alimentaire fixe, les micro-colonies sans couvain ne régulèrent plus les protéines, conduisant à la sur-collecte de ces dernières. Ainsi le couvain aide à la régulation nutritionnelle des ouvrières, ou bien ces dernières s'adaptent à leur présence.

Dans le troisième chapitre, j'ai examiné l'effet de pressions environnementales sur le comportement et les performances des bourdons en observant leurs choix nutritionnels à plusieurs températures. À température optimale (30°C), les bourdons composèrent un régime alimentaire favorisant leurs fonctions reproductrices ; alors qu'à basse température ils cherchèrent du sucre pour améliorer leur survie ; et à haute température, il se concentrèrent sur la prise en eau et/ou lipides.

Dans le contexte du fort déclin mondial des populations d'insectes, une meilleure compréhension du comportement nutritionnel des pollinisateurs est cruciale. Mieux comprendre le comportement des bourdons, commercialisés pour la pollinisation, permettrait d'améliorer leur activité de butinages et ainsi d'amoindrir la crise actuelle de pollinisation des plantes agricoles.

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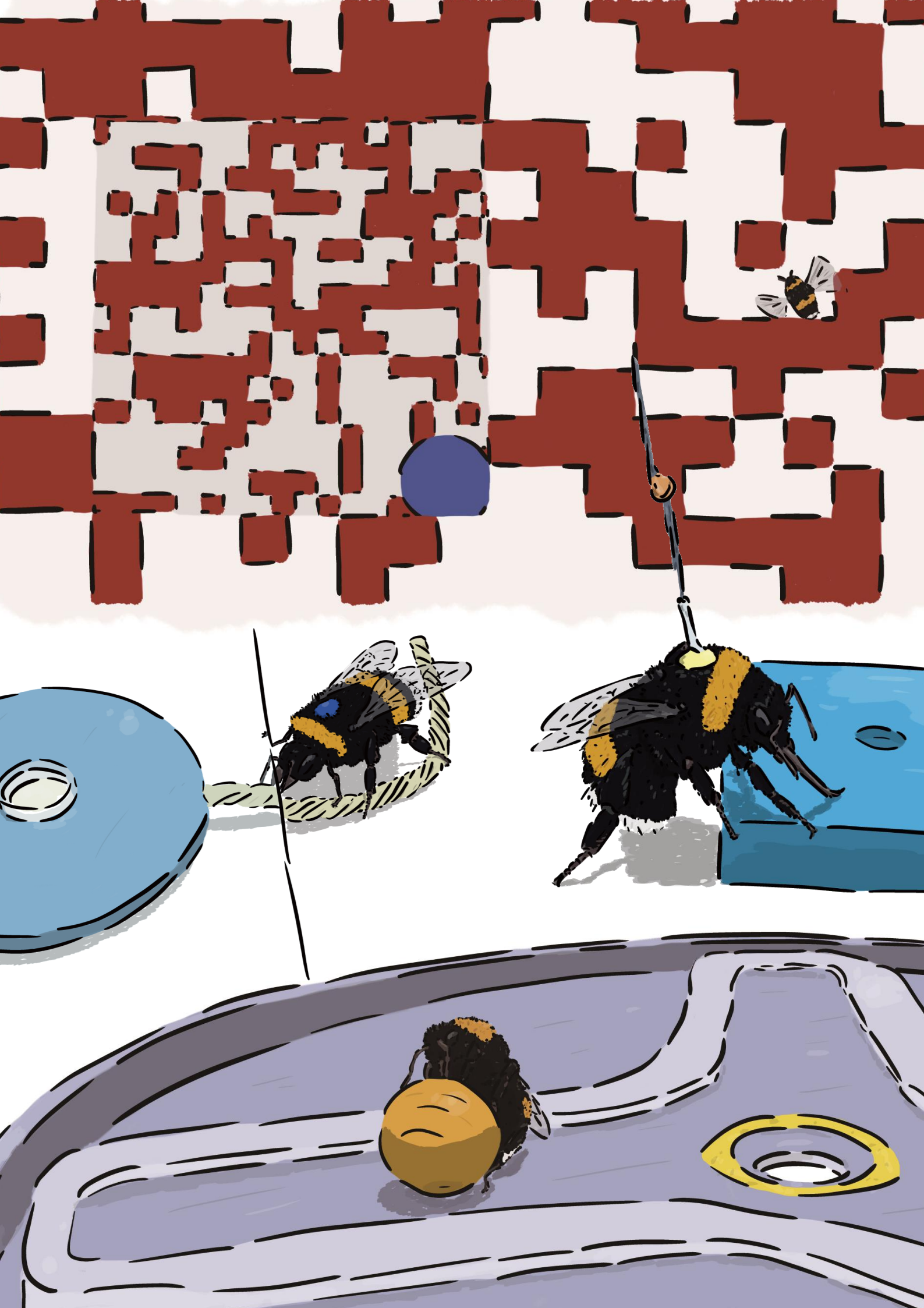
je serais plus là), Louis (qui a le bonus SeaofThieves, Blood Machine et Dune), Floflo (représente le meilleure M2 qui existe), Fares, Manon, Émilie, Alid, Yuan, Haiyang, Nour, Léo, Flora, Ben, Marco et Renaud. Une pensée pour Gérard, qui est un exemple de bonté. Il fait partie de ces personnes qui vous alpague durant des heures sur un sujet quelconque, mais avec plaisir, tout comme peuvent le faire Patrick ou encore Olivier. Merci à vous.

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[Cookie : I got your four basic food groups! Beans, bacon, whisky and lard?]

-- *Atlantide, the lost empire (2001)*



GENERAL INTRODUCTION

Studying animal nutrition with Nutritional Geometry

[Willy Wonka: Do you like my meadow? Try some of my grass! Please have a blade, please do, it's so delectable and so darn good looking!

Charlie Bucket: You can eat the grass?

*Willy Wonka: Of course you can! Everything in this room is eatable, even *I'm* eatable! But that is called "cannibalism," my dear children, and is in fact frowned upon in most societies.]*

-- Tim Burton, *Charlie and the Chocolate Factory* (2005)

Charlie in the Chocolate Room is now puzzled by the implication that everything is edible, and faces a tough dilemma: what to eat and in which quantity? In behavioural research, these critical feeding decisions have long been interpreted considering that individuals should eat most abundant or energetically rich foods (e.g., optimal foraging theory). So maybe Charlie would have to stick to the buttercups sugar candies that cover the ground. However, recent advances in nutritional ecology shows this is more complex. Animals, from worms, to molluscs, insects, reptiles, birds, and mammals, need to eat to develop, survive and reproduce. But they must do so by carefully choosing foods yielding them key nutrients in the right balance. As for Charlie, if he wants to avoid the fate of Augustus or Violet that eat too much chocolate and blueberry, he should pick a taste of marshmallow bushes, a snack of sweetmeat trees and a lick in the chocolate river.

These food choices and their consequences on animal fitness are best understood using models of nutritional geometry (Simpson and Raubenheimer, 2012). After demonstrating their efficiency first on locusts by visually confronting on a graph the intake of specific nutrients against others (Raubenheimer and Simpson, 1993), nutritional geometry models are now commonly used for studying animal nutrition across a wide diversity of taxa (drosophila: Lee et al. 2008a; beetles: Sun et al., 2022; ants: Dussutour and Simpson, 2009; honeybees: Paoli et al., 2014a; mice: Solon-Biet et al., 2014; cats: Hewson-Hughes et al. 2011; grizzlies: Coogan and Raubenheimer, 2016 ; humans: Wali et al., 2021).

Nutritional space and nutrient diversity

How does that work? In nutritional geometry models, the environment is defined by several food components (typically macro-nutrients, but not only). Foods are represented by “nutritional rails” each characterised by a ratio of the different food components they contain (Figure 1). These rails define a nutritional space in which animals can navigate (Figure 2.a.c). By eating more or less of each food, an individual can move its nutritional state, i.e., the amount and ratio of nutrients at a given time, in the nutritional space. The number of dimensions defining the nutritional space depends on the number of food components of interest for the animal, and on our ability to visually represent them, since adding dimensions significantly complexifies analyses. Usually, nutritional geometry studies confront major macronutrients by pairs such as proteins versus carbohydrates (e.g., in crickets, Maklakov et al., 2008; Figure 2.a) or proteins versus lipids (e.g. in seabird: Machovsky-Capuska et al., 2016; Figure 2.b). Yet, recent studies demonstrated more diverse interactions, by comparing more macronutrients together (e.g. carbohydrates, lipids and proteins in dogs, Hewson-Hughes et al., 2013; Figure 2.c) , combining them into groups (e.g., protein versus non-protein energy in gorillas, Rothman et al., 2011; Figure 2.d), and extending analyses to micronutrients (e.g. phosphorus versus calcium in chickens, Bradbury et al., 2014; Figure 2.e) or even non-nutritive components (e.g. marine debris in turtles, Machovsky-Capuska et al., 2020; Figure 2.f).

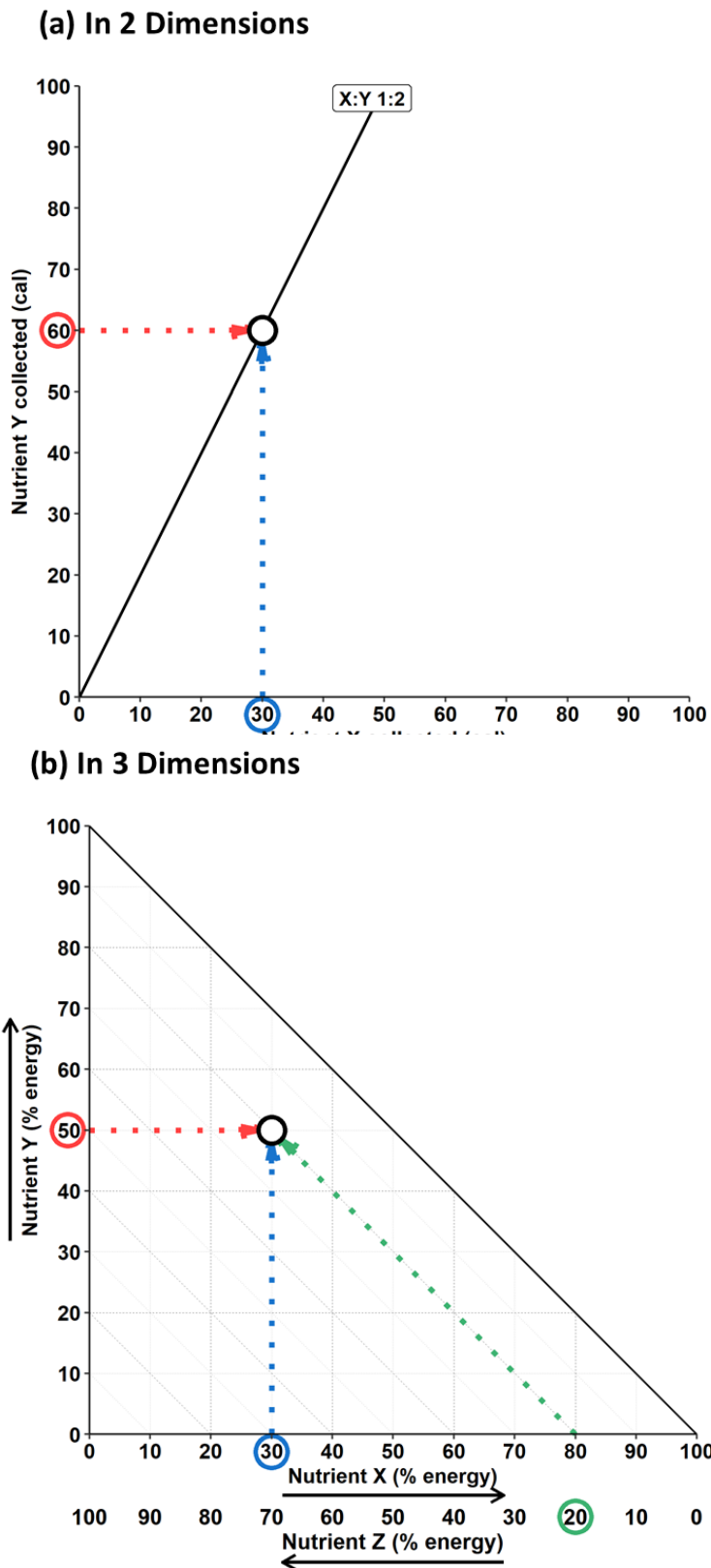
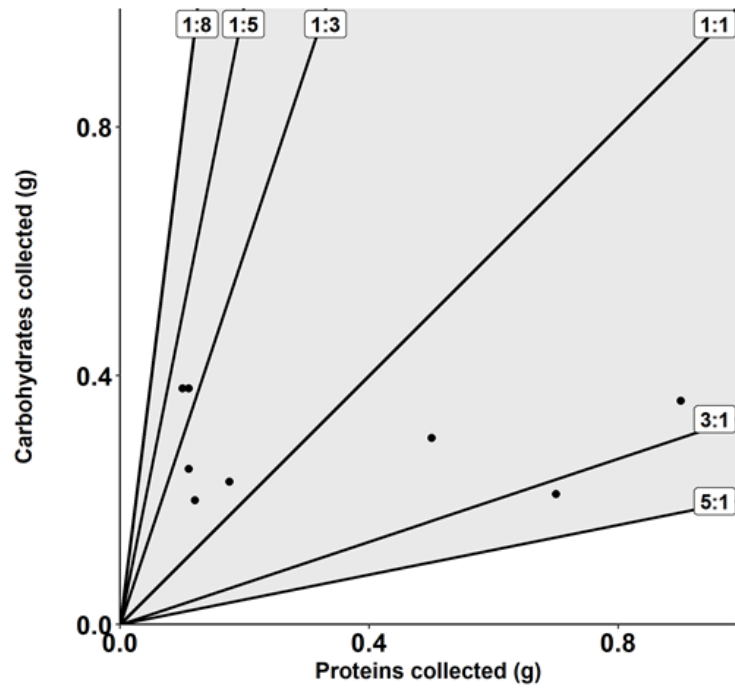


Figure 1: Theoretical examples of nutritional geometry models. (a) Nutritional spaces in 2 dimensions with collection of nutrient X versus nutrient Y in calories. Nutritional rail (black line) represents the ratio of nutrient X and Y in food. Nutritional state (white circle) corresponds to the amount of nutrient X and nutrient Y the individual collected, 30 (blue) and 60 (red) calories, respectively. (b) Nutritional spaces in 3 dimensions with the percentages of the three nutrients collected, using a right-angle mixture triangle (RMT) where nutrient Z is read diagonally from the most concentrated at the apex of the right angle to the least at the hypotenuse (Raubenheimer, 2011). White circle corresponds to either a food ratio or an individual nutritional state of 30% nutrient X (blue), 50% nutrient Y (red) and 20% nutrient Z (green). The amount of each collected nutrient cannot be directly represented using RMT.

(a) P:C in cricket (Maklakov et al. 2008)



(b) P:L in seabird (Machovsky-Capuska et al. 2016)

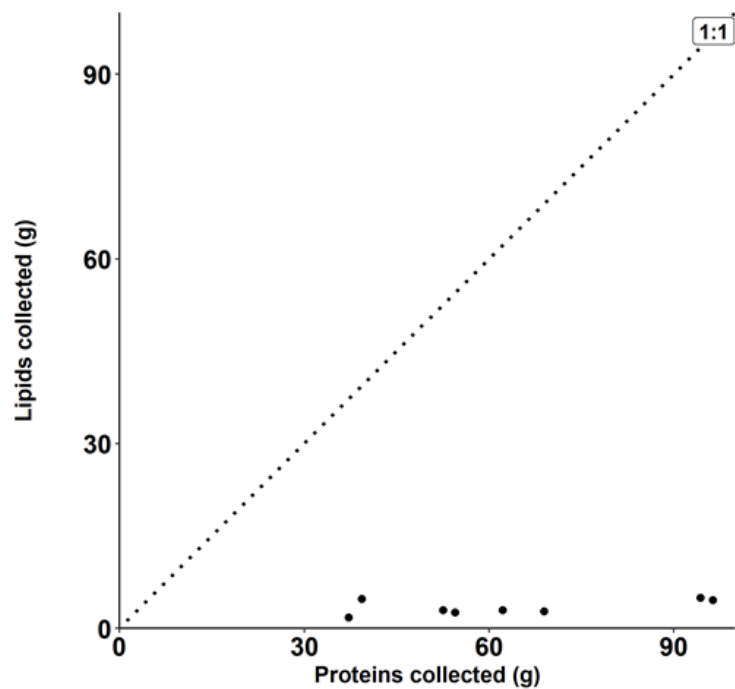
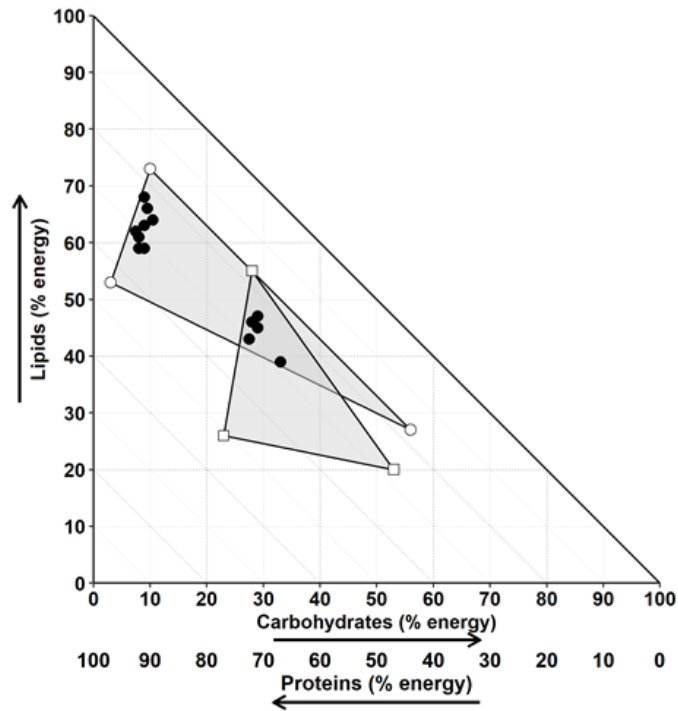


Figure 2: Examples of diverse uses of nutritional geometry. Nutritional spaces in 2 dimensions for (a), (b), (d) and (e), and in 3 dimensions for (c) and (f). In all the graphs, black dots correspond to individual nutrient intakes. **(a)** Proteins versus carbohydrates (P:C) collection in cricket (Maklakov et al., 2008). The nutritional spaces are represented in grey zones and are formed by their respective foods. Foods are represented by solid lines that correspond to the respecting ratio for each diet. **(b)** Proteins versus lipids (P:L) collection in seabirds (Machovsky-Capuska et al., 2016). The dotted line corresponds to the 1:1 ratio.

(c) C:L:P in dog (Hewson-Hughes et al. 2013)



(d) P:non-P in gorilla (Rothman et al. 2011)

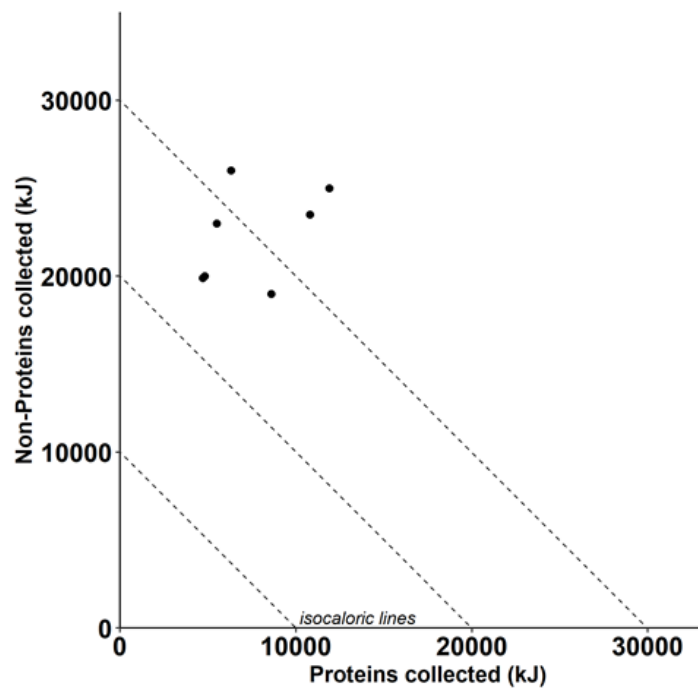
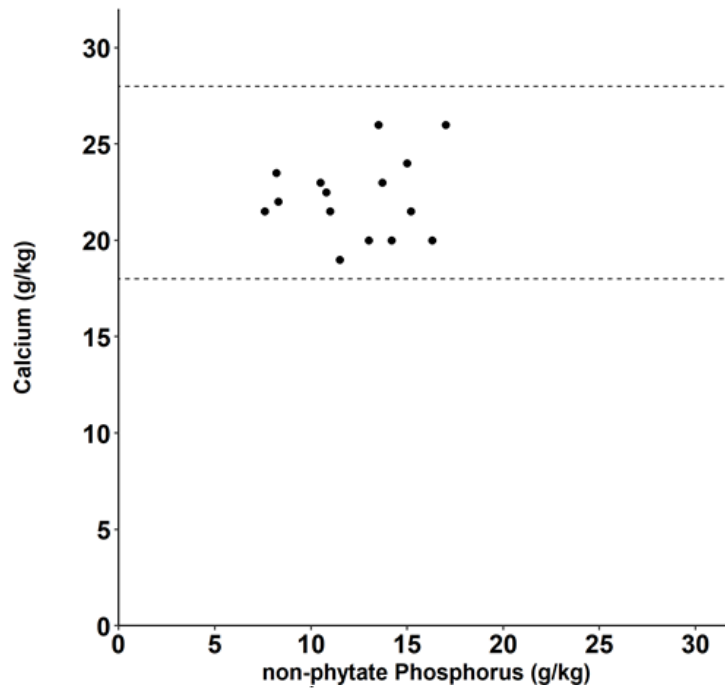


Figure 2: Examples of diverse uses of nutritional geometry. (c) Proportions of carbohydrates, lipids and proteins (C:L:P) eaten by dogs (Hewson-Hughes et al. 2013). Two nutritional environments are represented in grey zones and are formed by their respective foods (white symbols). (d) Protein versus non-protein (P:non-P) sources of energy in gorilla (Rothman et al., 2011). Dotted lines represent isocaloric distributions, where all points relate to the same energy intake.

(e) nPP:Ca in chicken (Badbury et al. 2014)



(f) Debris:non-P:P in turtle
(Machovsky-Capuska et al. 2020)

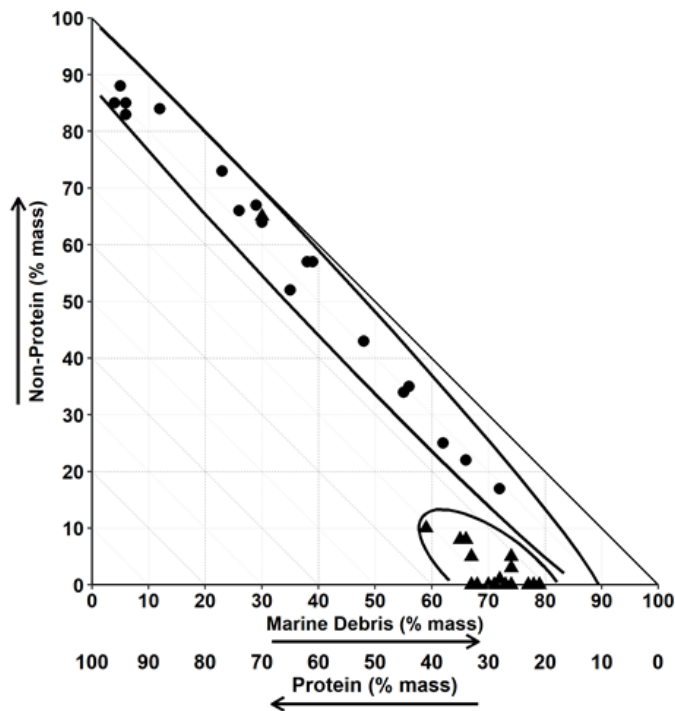


Figure 2: Examples of diverse uses of nutritional geometry. (e) Phosphorus versus calcium (nPP:Ca) intakes in chicken (Bradbury et al., 2014). Dotted lines delimit the interval in which the population regulates intake of a specific nutrient, here calcium. (f) Marine debris versus protein and non-protein (Debris:non-P:P) percent of mass ingested by turtle (Machovsky-Capuska et al., 2020). Ellipses correspond to nutritional niches, in which populations could persist by exclusively eating food with that nutrient composition.

Macronutrients in food: carbohydrates, lipids and proteins

What do animals need to eat? Macronutrients are chemical substances divided in three classes: carbohydrates, lipids and proteins, where each class contains different micronutrients (Table 1). For most animals, macronutrients are consumed in large quantities and provide brick elements and energy necessary for growth, tissue maintenance and reproduction (Pond et al., 2004; Cohen, 2015; Kraus et al., 2019b). By contrast vitamins and minerals, that are important for growth and enzymes production, are only needed in trace amounts.

Carbohydrates are the main source of quickly-mobilized energy. They can serve as a source of carbon for molecular synthesis or can be stored for the future. Since glucose can be synthesized from lipids or amino acids by gluconeogenesis (Miyamoto and Amrein 2017), some species can live without any sugar intake.

Proteins serve a wide range of biological functions, essentially as cell structure, enzymes, transport and storage, or receptor molecules. Proteins and free amino-acids are the principal source of nitrogen, and once assimilated, proteins are broken down and turned into different proteins. There are nine to ten amino-acids that cannot be synthesized and thus considered as essential depending on species (Table 1).

Lipids (hydrophobic molecules including fatty acids, sterols, glycerides, waxes and phospholipids) are a major, easily stored source of energy, and also serve as principal components of cell membranes, as well as nutrient transporters, defensive compounds, and precursors for pheromone and hormone synthesis.

Table 1: List of nutrients commonly used or essential in animals. Some of these can be nonessential or even toxic in some species, depending on each species' ability to synthesize or to digest and eliminate it (e.g., cellulose) (Pond et al., 2004; Cohen, 2015; Kraus et al., 2019b).

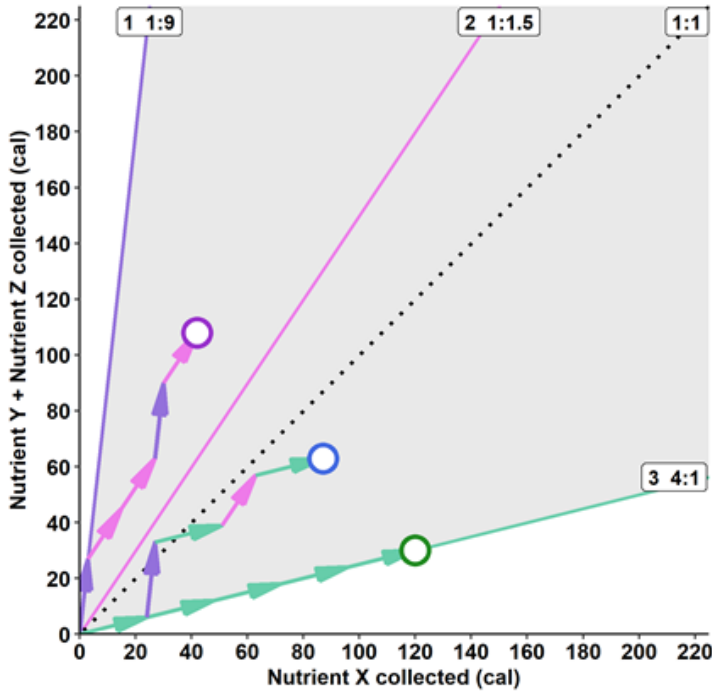
Proteins	Lipids	Carbohydrates	Vitamins	Minerals
Polypeptides	Sterols	Hexose	Water-soluble vitamins	Calcium
Glycoprotein	Cholesterols	Glucose	Ascorbic acid	Chlorine
Lipoprotein	b-Sitosterol	Fructose	Thiamine	Copper
Essential amino acids	Stigmasterol	Disaccharides	Riboflavin	Iron
Arginine	Campesterol	Sucrose	Pyridoxine	Magnesium
Histidine	24-methyl-cholesterol	Polysaccharides	Nicotinic acid	Manganese
Isoleucine	Phospholipids	Starch	Pantothenic acid	Phosphorus
Leucine	Fatty acids	Glycogen	Biotin	Potassium
Lysine	Linoleic acid	Cellulose	Folic acid	Sodium
Methionine	Linolenic acid		Choline	Sulfur
Phenylalanine			Cyanocobalamin	Zinc
Threonine			Inositol	
Tryptophan			Lipid-soluble vitamins	
Valine			Tocopherol	
			Vitamin A	

Reaching the intake target

How much do animals need to eat? Individuals need to eat food in order to acquire nutrients in amounts and balances that will maximize fitness traits such as development (Jang and Lee, 2018), metabolic health (Solon-Biet et al., 2015), survival (Piper et al., 2014) and reproduction (Maklakov et al., 2008). In nutritional geometry, these optimal nutrient mixes are represented as “intake targets” in the nutrient space (Figure 3). The intake target typically yields the highest average fitness by maximising the expression of several fitness traits whose optima are often located in different regions of the nutritional space. Consequently, this intake target varies as animals develop and age, investing first mostly in their growth and then in reproduction and survival. The challenge for any individual animal is thus to reach and maintain this changing intake target as well as possible (Simpson and Raubenheimer, 2012). As illustrated in figure 3, animals can do this in many ways, for instance by eating from a single nutritionally balanced type of food whose nutrient composition fits with the intake target (as for specialist species: green circles) (Behmer, 2009), or by combining intake from multiple food sources (each one away from the target) in a complementary manner (as for generalist species: purple and blue circles).

When neither nutritionally balanced nor complementary food sources are available in the environment, individuals cannot maximize their fitness by reaching their intake target, and thus have no other choice than making a compromise between over-ingesting or under-ingesting some nutrients (Figure 3.a). Feeding on an unbalanced diet for a prolonged period can move an individual’s nutritional state far away from its intake target and alter various fitness traits such as growth and reproduction. Note that using right-angled mixture (Figure 1.b) triangle has both its advantages and disadvantages because the simultaneous representation of three nutrients percentages does not allow us to know the quantities actually ingested. Examples of those advantages and disadvantages are shown in Figure 3.b.

(a) Intake target in 2 Dimensions



(c) Rules of compromise in 2 Dimensions

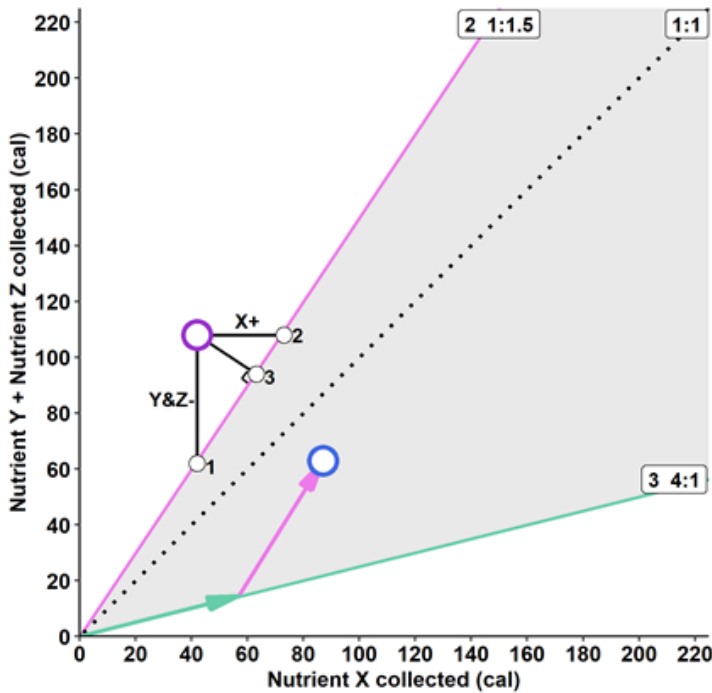


Figure 3: Intake target and rules of compromise in nutritional geometry. Intake target: (a) In 2D, representing nutrient X versus nutrients Y + Z collection in calories. Nutritional rails (coloured lines) represent the ratio of nutrients X and Y+Z in foods, while the dotted line corresponds to a 1:1 ratio. Grey zones correspond to the nutrient space delimited by the combination of food sources (1-3) made available, within which individuals can virtually move their nutritional state. Coloured circles represent nutritional intake targets (IT), the optimal states maximizing one or several traits. Arrows represent examples of collection paths to achieve each IT, with each colour corresponding to eaten food. The individual can reach its IT by eating on a balanced food only (food 3 contains the same nutrient ratio as the green IT) or by eating from two or more individually imbalanced but collectively complementary foods (food sources 1 and 2 for the purple IT, 1,2 and 3 for the blue one). **Rules of compromise:** (c) In 2D, the individual's nutritional state is represented by white dots and its intake target (IT) is represented by the purple circle. If the individual is limited to a unique unbalanced food, or if its IT is outside its nutrient space (grey zone), it has three options: (1) eat each food source less than necessary in order to regulate one nutrient (X) while having deficiencies in others (Y&Z-); (2) eat too much of one nutrient (X+) while regulating others (Y and Z); (3) make a compromise without fully regulating either nutrient, for instance by reaching the closest distance to the IT.

(b) Intake target in 3 Dimensions

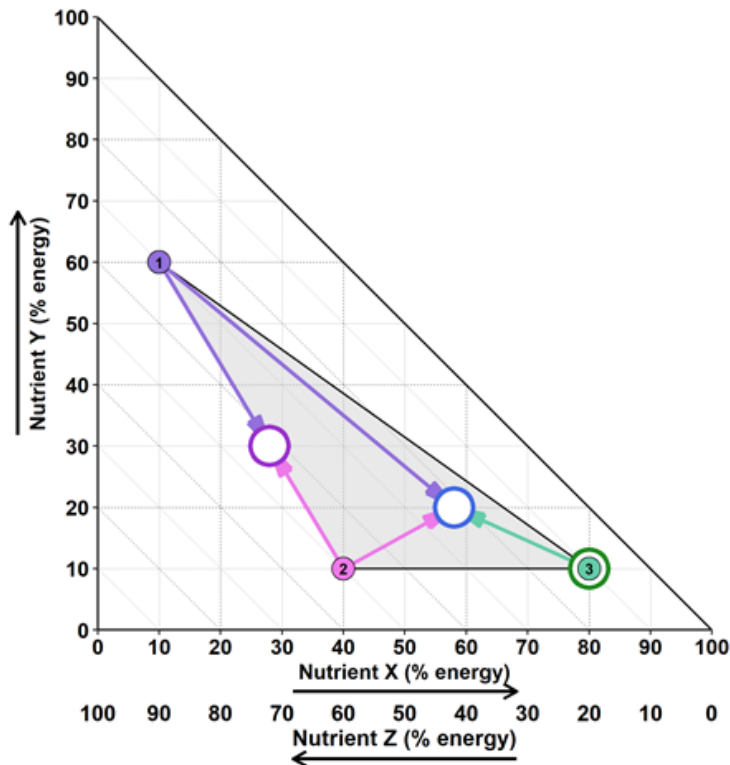
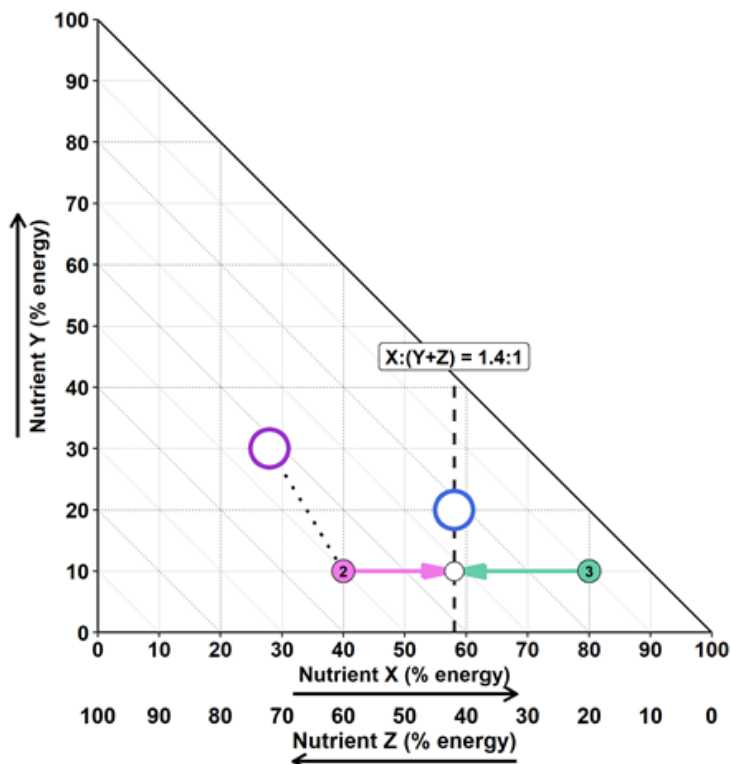


Figure 3: Intake target and rules of compromise in nutritional geometry. Intake target: (b) 3D-representation in a RMT, with the percentages of the three nutrients collected, where nutrient Z is read diagonally from the most concentrated at the apex of the right angle to the least at the hypotenuse. Grey zones correspond to the nutrient space delimited by the combination of food sources (1-3) made available, within which individuals can virtually move their nutritional state. Coloured circles represent nutritional intake targets (IT), the optimal states maximizing one or several traits. Arrows represent examples of collection paths to achieve each IT, with each colour corresponding to eaten food. The individual can reach its IT by eating on a balanced food only (food 3 contains the same nutrient ratio as the green IT) or by eating from two or more individually imbalanced but collectively complementary foods (food sources 1 and 2 for the purple IT, 1,2 and 3 for the blue one). **Rules of compromise: (d)** In 3D-representation in a RMT, visualizing this unbalanced scenario has the inconvenient of informing only on the closest diet to IT (here, source 2/purple IT), without information on quantities of over- or under-ingested nutrients. However, where in 2D (c), the individual reaches the blue IT by intake of food sources 2 and 3, the 3D visualisation (d) allows us to remark that only the intake of X fits the IT (dashed line: ratio $X:(Y+Z)$) while the proportions of Y and Z do not fit.

(d) Rules of compromise in 3 Dimensions



The nutritional challenge of social insects

[A monster, moreover, complete! Its garrets, as it were, a head full of knowledge and genius; its first storeys stomachs repleted; its shops, actual feet, where the busy ambulating crowds are moving. Ah! what an ever-active life the monster leads! Hardly has the last vibration of the last carriage coming from a ball ceased at its heart before its arms are moving at the barriers and it shakes itself slowly into motion. Doors open; turning on their hinges like the membrane of some huge lobster, invisibly manipulated by thirty thousand men or women.]

-- Balzac, *Ferragus*

This is how the French writer Balzac described Paris, and how he might have done for an insect colony. The capital city was depicted as a living monster, and an insect colony too could be characterized as a unique individual, a “superorganism”. Social ants, bees, wasp and termites live in colonies of hundreds or thousands of individuals, where the reproductive, foraging and nest-building tasks are divided. The workers are commonly sterile, and cooperate to breed the larvae, producing colonies with overlapping generations of individuals (Hölldobler and Wilson, 2009). Using nutritional geometry, several studies have characterised the diet regulatory strategies in many animal species, from insects to mammals (Simpson and Raubenheimer, 2012). Recently, this approach was extended to the study of group nutrition where individual feeding decisions are socially influenced (Lihoreau et al., 2015). Indeed, in contrast to an individual animal, these superorganisms have a mean nutritional intake target, which is the sum of all members’ one, and varies conjointly with colony composition.

Disparate nutritional needs in the colony

In social insect colonies, adult workers essentially require carbohydrates as a source of energy serving for their own survival as well as nest maintenance, thermoregulation, guarding and foraging (ants: Feldhaar, 2014; honey bees: Pirk et al., 2010; Paoli et al., 2014a; bumblebees: Stabler et al., 2015, termites: Poissonnier et al., 2018). Reproductive individuals need proteins, because gonad development and egg-laying is

a nitrogen-limited activity thus depending on protein intake (honey bees: Altaye et al., 2010; Pirk et al., 2010; bumblebees: Tasei and Aupinel, 2008a; Moerman et al., 2017; wasps : Schmidt et al., 2012). Males have less fat content, investing less in lipid storage compared to gynes and workers, due to their shorter lifespan (e.g., Schmidt et al., 2012). The larvae drive foragers' food collection toward the nutrients they require for their own growth and physiological maturation (ants: Dussutour and Simpson, 2009; Feldhaar, 2014; bumblebees: Moerman et al., 2016). In some species, the amount and quality of proteins fed at the larval stage can determine the future caste of the individual (honey bees: Maleszka, 2018; Wright et al., 2018; wasps: Schmidt et al., 2012).

Foraging in coordinated groups

In such societies, only workers specialised in foraging tasks leave the nest, solely or in groups, to collect food outside and take it back to the nest. They are for the colony in Balzac's words: [its shops, actual feet, where the busy ambulating crowds are moving]. By specialising in particular resources or sharing information about their environment, foragers could increase their probability of finding the required nutrients in a short amount of time by partitioning their foraging area or nutritional resources (Grüter et al., 2014; Leadbeater, 2014; Giraldeau and Caraco, 2018; Dubois et al., 2021). And as a result of diversity in behavioural profiles among individuals, foragers manage to maintain a continuous food collection in changing environments (Jandt et al., 2014; Jeanson and Weidenmüller, 2014). In bumblebees, foragers that make fast decisions have a greater chance to make mistakes, compared to foragers that take times. However, fast foragers have a greater nectar income than slow foragers in rich environments, where resources are present in high quantity. On another hand, the seconds types of foragers outperform the first in poor environments where resources are spread and thus foraging errors are severely punished (Burns and Dyer, 2008; Muller and Chittka, 2008).

Moreover, from simple inter-individual interactions can emerge a collective behaviour increasing dramatically foraging dynamics and efficiency (Conradt and Roper, 2005). Ants are a perfect illustration of coordinated foraging strategies, as some species lay a pheromonal trail before and after finding a food resource (Detrain and Deneubourg,

2008; Morgan, 2009). Like the *Little Thumb* from Charles Perrault's story, ant foragers mark the ground every few steps to remember their path. The pheromonal trail plays a major role in nutritional cue-sharing and decision process - the latter because a profitable resource will increase the frequencies of pheromone drop, and the former because the more pheromone there is in a trail, the higher the probability for an ant to use it. Those positive feedbacks influence, in a non-linear manner, other foragers' responses to a particular trail leading to the rapid exploitation of the resource it leads to. Similar collective behaviours occur in honeybees, with the peculiarity that cue sharing takes place directly in the hive. The *waggle dance* (first described by von Frisch) allows foragers, by making eight-shaped vibrating movements, to share the precise direction, distance and affordability of flowers (Frisch, 1967; Couvillon, 2012). The more intense the vibrations, the higher the chance for other foragers from the colony to follow the corresponding indications (Detrain and Deneubourg, 2008).

Foods in nest: from storage to nutritional cues

How do foragers know what to collect? Harvested food enters colonies after a cascade of interactions from foragers to in-nest workers, where it is processed and given to other adults and the larvae (Wright et al., 2018). These nutritional transfers involve either mouth-to-mouth liquid food exchanges (trophallaxis) or food storage.

food stores, as well as colony wastes (e.g., wax, brood combs, pellets of pollen, rejected foods, dead bodies), can serve as sources of information for foragers to know which nutrients are lacking or in excess (Dreller et al., 1999; Vaughan and Calderone, 2002; Hendriksma and Shafir, 2016). In honey bees, these nutritional assessments can be transferred via trophallactic pathways along chains of individuals, from in-hive workers to foragers (Schmickl and Crailsheim, 2004). Immobile larvae, like young birds in a nest, call for food through pheromonal emissions that inform workers on the developmental stage and nutritional needs, so that they can adapt their food choices (ants: Dussutour and Simpson, 2009; honey bees: Dreller et al., 1999): [Its garrets, as it were, a head full of knowledge and genius].

The case of bumblebees

Since their domestications in the 1980s for greenhouse pollination, bumblebees have gained an important economic value (Velthuis, 2002; Velthuis and van Doorn, 2006). Together with other insects, they provide a pollination service estimated to \$361 billion worldwide (Lautenbach et al., 2012). This colossal estimate raises questions about the sustainability of agricultural activities in the context of the current global collapse of insect biodiversity (Goulson et al., 2015; 2019; Wagner et al., 2021). Key crops like tomatoes and potatoes require vibrations on their anthers to remove their pollen grains (De Luca and Vallejo-Marín, 2013; Vallejo-Marín, 2019), a behaviour called “buzz-pollination” that is not found in honeybees, and worth pollination service estimated to €12 million per year only for tomatoes (Velthuis and van Doorn, 2006). Therefore, as pollination is linked to food searching in bees and animals in general, a main question related to understanding bumblebee nutrition is whether, and how, pollination could be improved by manipulating bees’ nutritional behaviour?

Hijacking bumblebee’s colony lifecycle to support science

Most of bumblebee’s species live in colonies of hundreds of workers and a single queen (Goulson, 2010). Besides, their resilience to captivity and their large body sizes that facilitate tracking, bumblebees are excellent research models for numerous fields, including nutritional ecology.

Colony lifecycle is initiated with the nest foundation: a single queen emerging from hibernation in spring will search a decent nest site to lay its first batch of eggs (Goulson, 2010). The new queen collects the amounts of nectar and pollen required for colony initiation, i.e., about 6g of pollen (with 1g of protein in it) and 50g of sugar (Rotheray et al., 2017). She broods the eggs by maintaining an thoracic temperature of 37-39°C, which results in 30-32°C on brood cells (Goulson, 2010). This is a critical stage when stressors could be detrimental for the fate of the colony (Elston et al., 2013; Baron et al., 2017).

When the first workers emerge, they subsequently help the queen in nursing and replace her for foraging. From then on, nest growth accelerates until a 'switch point' where the queen starts laying eggs developing into fertile offspring. The ratio between workers and larvae then changes in favour of the latter (Duchateau et al., 2004; Goulson, 2010). This is a conflictive period where workers with a greater degree of ovarian development fight to lay their own eggs (Foster et al., 2004). As in other social insect species, their offspring is haploid and yields males. In parallel, new queens forage to build up lipid reserves to sustain the colony during winter. Then, males and females will fly to find their mates, a reproductive encounter that ends in the drones' death and hibernation for the young princess. After what the new queen will revive this arduous cycle for one more year.

Nevertheless, thanks to their domestication via breaking queens' hibernation, controlling mating, food sources and climate in rearing rooms, bumblebees could now be reared in the laboratory all over the year. In addition, the use of queenless microcolonies allow more replicates by dividing a mother colony with a single queen (Tasei and Aupinel, 2008b). All this has led to a significant increase in the number of studies on bumblebees over the last decades (Lihoreau et al., *in rev*).

Nutrient flow in a bumblebee colony

Bernd Heinrich described, in his book *Bumblebee Economics*, bumblebee colonies as factories that launch on the market queens and drones (Heinrich, 1979). We recognize the closeness of his metaphor, since nutrition includes every process in the factory, or here the colony, by which nutrients are transformed to provide proper functioning and reproduction to a living being, for the colony that is by sending fertile individuals in the environment. And because "nothing is lost, nothing is created, everything is transformed", and among all, nutrients in bumblebee's colonies are no exception to the rules; he described a flow diagram of protein and energy movement through a bumblebee colony that implies nutritional mechanisms similar for all social insect colonies (Heinrich, 1979). A revisited version is illustrated and detailed below (Figure 4), from nutrients' income to loss, through their transformation.

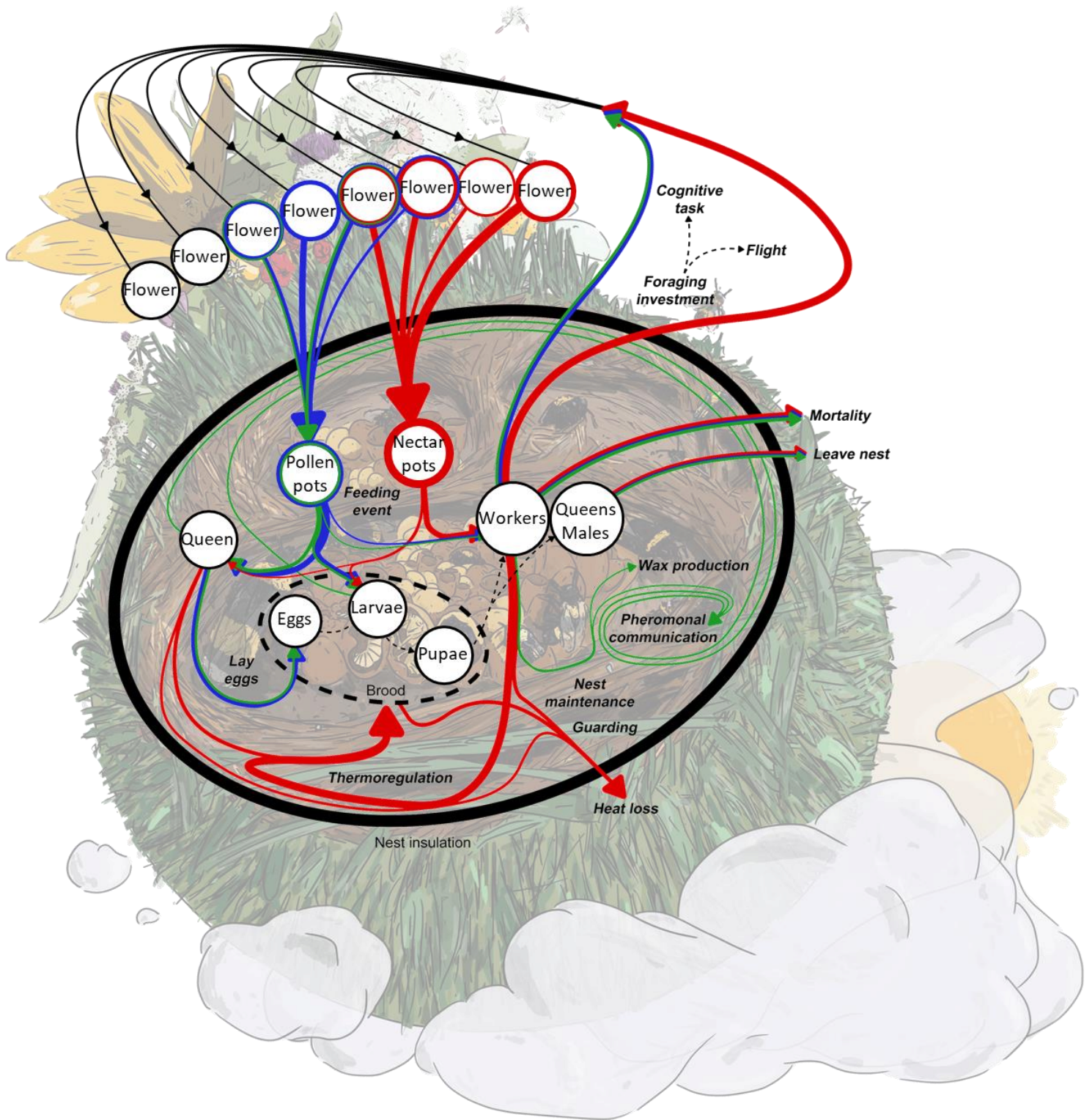


Figure 4: Nutrient flow through a bumblebee colony. Flow diagram of carbohydrates (red arrows), proteins (blue arrows) and lipids (green arrows) through three major steps in the bumblebee colony: nutrients income, transformation, and loss. See main text for detailed descriptions of each step. Adapted from Heinrich (1979).

Nutrient income: flowers resources and foraging cost

Foragers visit flowers to collect nectars and pollens, the only food resources bees exploit to acquire essential nutrients. While foraging, they store up to 60 to 120 μl of nectar (depending on the individual's size, Goulson et al., 2002) in their crop, and/or pollen in their pollen basket (corbiculae, in the hind legs), that they can carry back to the nest.

Nectar mainly provides carbohydrates, water and some free amino-acids, but also trace amounts of lipids, inorganic compounds, vitamins and plant secondary metabolites (Baker, 1977; Nicolson and Thornburg, 2007; Nicolson, 2011). However, the role of those micro compounds in nectar is not clear, and could mostly serve as stimulant or aversive agent, such as for amino-acids (Nicolson and Thornburg, 2007). Sugar concentration in nectar can range from 10 to 70% (w/w) in nature, but mostly are comprised within the range of bees preference (Kim et al., 2011), between 30 to 60%. Pollen is the principal source for proteins, lipids, vitamins and minerals (Roulston and Cane, 2000). Proteins are 2-60% dry mass in pollen grain, while lipids represent 1-25% dry mass (Wright et al., 2018; Vaudo et al., 2020). Starch and fibres, non-digestible by bees, represent a large part of available carbohydrates (Roulston and Cane, 2000).

Yet, foragers have some investments to make before expecting nutritional yield: flight is costly, demanding about 0.25 calories per minute for a medium bumblebee. Moreover, it requires keeping the thoracic temperature between 30 and 44°C, so that the foragers have to warm up when air temperature is lower, which can increase the caloric demand up to 200% at 5°C (Heinrich, 1979). As chance not always rewards bees, flowers could be hard to locate or found already emptied by previous pollinators. Foragers need to trade-off between minimizing flight distance and prioritizing high-reward intake (Lihoreau et al. 2011), at the risk of consuming all the nectar stored in their crop as fuel for flight before returning to the nest.

Moreover, pollen foraging requires that bees learn complex behaviour to collect the few grains available in each stamen. A study showed that bees need at least 300 visits on poppy flowers to achieve an optimal pollen collection, three times longer than to master nectar collection (Raine and Chittka, 2007). Besides, located and remembering flower locations, those cognitive processing adds another cost to foraging activity, as learning

and memory are not energy-free operations (Burns et al., 2011). Malnourished insects demonstrated reduced performance in various cognitive tests (unbalanced proteins to carbohydrates ratio in drosophila: Lihoreau et al., 2016; fatty acid deficit in honeybee: Arien et al., 2015; 2018), suggesting the nutrients requirement in these tasks. These results are consistent with decreased survival when insects were conditioned on learning assays (drosophila: Frederic and Kawecki, 2003; 2004; wasps: Kishani Farahani et al., 2021).

Those foraging costs reveal the critical challenge that bees face when searching for flowers, as they have to choose between food sources to fulfil a precise nutrient needs for all of the entire colony.

Nutrient transformation: brood production and development

In the bumblebee colony, nutrient incomes are then transformed and processed to produce new adult workers, males and queens. Pollen intake elicits egg-laying by the queen: it deposits eggs in clumps coated in wax, which hatch within 3-4 days (Alford, 1971). Young larvae then undergo four instars over 10-14 days before entering pupal stage, enclosed in a silk cocoon for another 10-14 days to finally emerge as imagoes (Alford, 1975). In total, full development lasts 3-5 weeks, depending on caste, temperature and food supply.

Pollens rich in protein enable eggs to be laid earlier and in greater numbers (Génissel et al., 2002; Watrous et al., 2019) and increase larval growth rate (Tasei and Aupinel, 2008a; Moerman et al., 2016). As such, blends of pollens have shown to give at least equal if not better performance than mono-diet (Ryder et al., 2021), highlighting the importance of a nutritional balance in food (Vanderplanck et al., 2014; Moerman et al., 2017). A new method using lanthanides to mark nutrients and measure their amounts left after digestion was used in bumblebee larvae (Řehoř et al., 2014), leading to an estimate of 0.52 mg proteins and 4.43 mg sugars required (per milligram of body mass) to produce a worker (Macháčková et al., 2019), corresponding an ideal diet ratio of 1:8.5 P:C. When feeding larvae, queens and workers give about one microliter of food, containing about 30 µg protein and 460 µg sugar (Pereboom, 2000). This estimate from

previous studies (Pereboom, 2000; Macháčková et al., 2019) implies that a larvae needs to receive 10-20 such food portions to undergo its full development.

There is no difference in the food given to the larvae that explains reproductive caste differentiation in bumblebees, as observed in honey bees with royal jelly (Pereboom, 2000; Wright et al., 2018). Actually, it is not clear how reproductive division of labour is established in this species, but it has been suggested that a larval pheromone stimulates feeding by the workers during longer time, and thus extends their development (Cnaani et al., 1997; Goulson, 2010). By contrast, division of labour among workers is correlated with body size which, generally for all castes, depends on the position of larvae within the nest that determines developmental factors such as temperature and food received (Ribeiro 1994; Goulson 2010).

Finally, bumblebees produce wax essentially from lipids (Tulloch, 1970; Rottler et al., 2013) to coat egg- and larvae-cells, as well as covering the nest and maintain an optimal temperature for brood development, around 30°C (Heinrich, 1979; Gürel and Gösterit, 2008; Nasir et al., 2019; Wynants et al., 2021).

Loss of nutrients: workers' activities, departures of fertile individuals and mortality

Besides covering the nest with wax, workers warm the nest by non-flight thermogenesis that requires a certain amount of carbohydrate energy (Heinrich, 1979). A few workers allocate about 1.38 cal.min⁻¹ to maintain optimal temperature on a small lump of brood (380mm²) at 25°C in laboratory conditions (Livesey et al., 2019). Thus, if relying only on the sugar contained in its crop, hypothetically an average worker could contribute to thermogenesis for up to 130 minutes without any energy intake (based on an estimated sugar concentration of 50% w/w). On the opposite, an increase in temperature and/or carbon dioxide will elicit a fanning response from the bees, which impedes the ambient temperature to exceed the lethal limit for the brood (35°C, Heinrich, 1979; Weidenmüller et al., 2002; Gardner et al., 2007). As for warming the nest, fanning is an energetic costly activity, and response thresholds vary between individuals, with the majority of workers fanning when the temperature exceeds 32°C (Weidenmüller, 2004; Wynants et al., 2021).

In-nest workers share other energy-consuming tasks such as larval feeding (Pereboom et al., 2003; Jandt et al., 2009), or nest guarding (Jandt et al., 2009; 2012) and corpse removal (Walton et al., 2019) that prevent predators and parasites incursion, respectively (Goulson et al., 2018). Besides, immune responses, important against potential pathogen attack, require some protein expense (Riddell and Mallon, 2006; Lee et al., 2008b; McMenamin et al., 2018). Coordination between all those tasks is required within the colony, which relies partly on pheromonal communication in bumblebees (Ayasse and Jarau, 2014; Stökl and Steiger, 2017). Among those volatile compounds, synthesized essentially from fatty acids (Granero et al., 2005; Yew and Chung, 2015), some are known to elicit foraging (Granero et al., 2005; Molet et al., 2008) or larval feeding (Pereboom et al., 2003; Boer and Duchateau, 2006).

Finally, the ultimate goal of all this burden activity is the departure of new queens and males that are about to copulate with mates from different colonies and found new nests (Goulson, 2010). Although this is an investment in genetic renewal and spread, for the colony itself as an entity at a definite time, it counts as direct nutritional loss, just like the death of workers. Now, precise nutritional investment on development of each individual is highly variable and remains to be calculated or modelled.

Nutrient balancing in bumblebee colonies

Over the past years, bumblebees have emerged as model species for the study of the nutritional ecology of bees. Nutritional geometric experiments on small groups of bumblebees demonstrated, using semi-natural or artificial food, their ability to non-randomly select between foods, nutritional (Stabler et al., 2015; Vaudo et al., 2016a,b ; Kraus et al., 2019a). These laboratory studies showed that bumblebees converge onto specific ratios, either proteins:carbohydrates (Stabler et al., 2015) or proteins:lipids (Vaudo et al., 2016b). Nonetheless, these ratios would vary across studies based on different diets, because the nutrient sources could influence nutritional intake. A previous study observed that bumblebees converged to a ratio of 1:149 P:C while fed with caseinate-containing diets, but changed toward 1:560 P:C when casein was replaced by free amino-acids (Stabler et al., 2015).

Recently, nutritional studies on bumblebees highlighted the importance of lipids in regulating pollen collection (Vaudo et al., 2016a,b; Ruedenauer et al., 2020). Bumblebees showed a preference for pollens or artificial diets varying in P:L, richer in proteins than lipids (Vaudo et al., 2020). This demonstrates the importance of studying all three macro-nutrients simultaneously to better understand the foraging dynamics of bumblebees and their flowers species preference.

New questions tackled in my thesis

The aim of my CIFRE thesis was to better characterise the nutritional behaviour of bumblebees and its consequences for fitness, in order to identify potential nutritional needs and artificial diets that could be used to enhance pollination for crop production. I hypothesised that I could manipulate the nutrition of commercial bumblebee colonies in such a way that would increase visitation rates on targeted plants, thereby augmenting their pollination success.

To reach this aim, and to fill the knowledge gaps identified above, I conducted a comprehensive analysis of bumblebee nutritional requirements under various social and ecological conditions, using the experimental and theoretical approaches of nutritional geometry. My thesis is composed of three chapters.

Chapter 1 describes the perception of nutrients and the nutritional decisions by individual bumblebees. Nutrient detection occurs via gustatory sensillae mainly found on their antennae, mouthparts and tarsi (de Brito Sanchez, 2011; Bestea et al., 2021). Gustatory substances penetrate the sensillae through their pore or papilla located at the apex, and then contact the dendrites of up to five gustatory receptor neurons (Mitchell et al., 1999). Mechanoreceptor neurons can also be found at the base of the sensillae, and might evaluate the position and density of the food (de Brito Sanchez, 2011). Bumblebees can detect various nutrients in food, such as carbohydrates (Mommaerts et al., 2013), proteins (Ruedenauer et al., 2016; 2019) and lipids (Ruedenauer et al., 2020). Sensing proteins and lipids in food is based on amino acids and fatty acids, since proteins and most fat molecules are too large for gustatory receptors (Solms, 1969), and amino acids positively correlate with the total protein content (Weiner et al., 2010; Ruedenauer et al., 2019b). But what drives food choices in individual bees, from the

perception of those macronutrients to the choice made between diets across individual variations in nest as well as in foraging context, remains unclear. Here I studied how the perception of nutrients and the nutritional decisions varied between individual bumblebees. I speculated that bumblebees were more attracted by carbohydrates compared to lipids and proteins, and nutritional perception and regulation would be influenced by body size, as for task division in colony (Goulson et al., 2002).

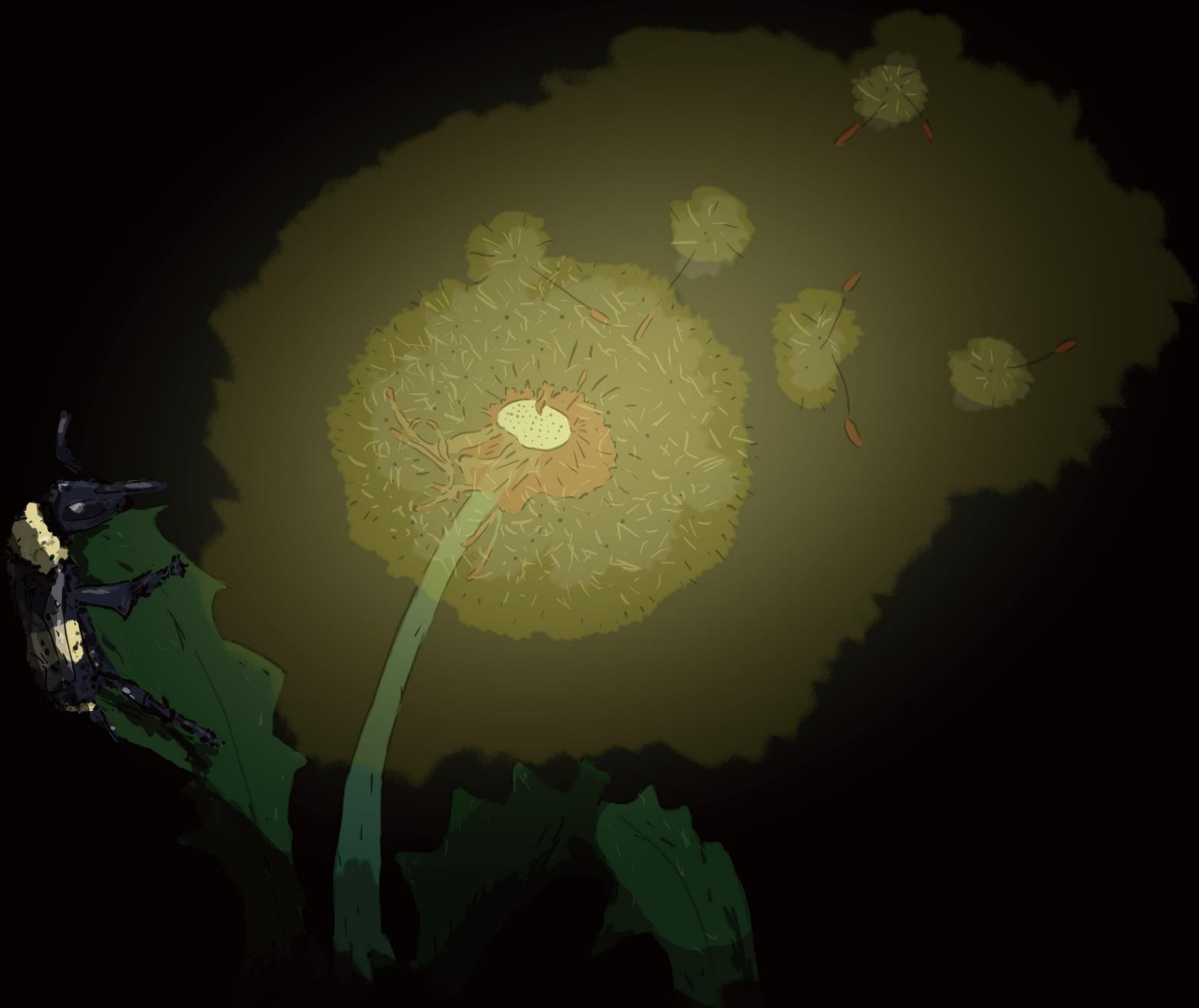
Chapter 2 describes the influence of brood on collection decisions by bumblebee colonies. The colony lifecycle over the seasons could influence foraging bees' food preference, as the colony composition and activities theoretically affect nutritional needs. Foragers prompt each other to go outside with recruitment pheromone and buzz, especially when food stocks are low (Cartar and Dill, 1990; Molet et al., 2008). The fact that starved larvae are more often fed than others (Smeets and Duchateau, 2001; Pereboom et al., 2003) and that workers discriminate between cuticular chemicals from starved larvae and fed larvae (Boer and Duchateau, 2006) suggest a larval pheromone used as a "hunger signal". Here I studied how brood could influence nutritional decisions made by workers. I hypothesized that brood presence would drive workers behaviour toward proteins collection to fulfil nutritional needs for their own development and maturation.

Chapter 3 describes the effect of temperature and nutrient shortage on nutrient collection by bumblebee colonies. Climate does not only directly disrupt insect condition, but influence the flowers availabilities and their resources qualities (Høye et al., 2013; Miller-Struttman et al., 2015; Descamps et al., 2021b), already undermined by human activities through habitat fragmentations and large monocultures (Howard et al., 2003; Goulson et al., 2015; St. Clair et al., 2020; Flowers et al., 2020). Unbalanced diet and malnutrition have detrimental effect on bees community (Klein et al., 2017), from cognition (Arien et al., 2015; 2018), immune response (Di Pasquale et al., 2013), to

larvae maturation (Moerman et al., 2016) and death (Vaudo et al., 2016b; Ruedenauer et al., 2020). Climate change already drove the global decline of various bumblebees species (Kerr et al., 2015; Martinet et al., 2015; Soroye et al., 2020). As temperature and nutritional stress conjointly crunch bumblebees' health, understanding how their effects interact, and if/how bees adapt to them, is crucial (Clissold and Simpson, 2015). Here I studied the effect of three environmental temperatures on bumblebee nutritional decisions. I suggested that workers would adapt their nutrient collection to the temperature, prioritizing their own survival when constrained to sub-optimal temperature for nest growth.

[Thumper The Rabbit: Eating greens is a special treat. It makes long ears and great big feet. But it sure is awful stuff to eat.]

-- *Bambi* (1942)



CHAPTER 1

VARIABILITY IN NUTRIENT PERCEPTION,
REGULATION AND SPECIALIZATION BY
INDIVIDUAL BUMBLEBEES

CHAPTER 1: Variability in nutrient perception, regulation and specialization by individual bumblebees

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Summary:

- Here we studied what drives food choices in individual bees; factors such as the macronutrient content, the age or caste of the workers, or social influences in foraging.
- We confirmed that carbohydrates induced more appetitive response than lipids and proteins in food.
- All workers tended to have a similar nutrient collection target irrespective of age and body size, while queens collected more protein-rich diet.
- Foragers displayed a generalist behaviour by visiting from multiple various artificial flowers to achieve their common intake target.
- Understanding how bumblebees sense the different nutrients in food and how they regulate their collection gives us insights on their nutritional adaptations through varying social and environmental context.

Key words: bumble bees; artificial diets; nutrient perception; nutritional regulation; foraging

Chapter Introduction

Whether it is a forager flying from flower to flower, or an in-hive worker eating nectar and pollen stocks in the nest, for an individual bee the question remains the same: what drives its food choices to achieve a specific nutrient balance? When and why does a bumblebee decide not to collect a specific type of food because it contains too much or too little of a given nutrient? Because there is a high degree of variation in the quantity and balance of micro- and macronutrients found in food resources (Roulston and Cane, 2000; Somerville, 2001; Petanidou et al., 2006; Weiner et al., 2010), the nutritional decisions are complicated and critical. As for the individual itself, a nutritional imbalance would lead to detrimental effects on fitness-related traits, including survival (Paoli et al., 2014b; Vaudo et al., 2016b), immune responses (Di Pasquale et al., 2013) and cognition (Arien et al., 2015; 2018; Muth et al., 2018). But it is also the case for the whole colony, where a correct nutrient income is necessary for queen egg production and larval development (Tasei and Aupinel, 2008a; Moerman et al., 2016; 2017). In this chapter, we asked what drives food choices in individual bees, from the perception of nutrients in food, to the choice made between diets across variations in age and caste, as well as within a social foraging context.

Bumblebees sense carbohydrates, proteins and lipids in food

Bumblebees can detect various nutrients in food and adapt their collection to their needs (Ruedenauer et al., 2016). In particular, they can detect carbohydrates (Mommaerts et al., 2013), proteins (Ruedenauer et al., 2016; 2019a) and lipids (Ruedenauer et al., 2020), as shown using different protocols. For example, a previous experiment showed the responses of bumblebees to the three main sugars found in nectars: sucrose, fructose and glucose (Mommaerts et al., 2013). Briefly, harnessed bumblebees performed a sensitivity test where the experimenter touched their antennae with a toothpick previously dipped in an increasing concentration of either a sucrose, fructose, or glucose solution. If the bumblebees extended their proboscis, this meant they perceived the nutrient concentration and were motivated to consume it. A

concentration of 5.5% (w/v) of either fructose or glucose solution was enough to induce a proboscis extension of half of the tested bumblebees, thus revealing a substantial level of motivation for rather low contents of these sugars, while such level of response was achieved at a much higher concentration (40%) for sucrose. However, when bumblebees were let to forage on artificial flowers and thus freely assess the food (with their antennae and tarsi) before collection, foragers showed a preference for sucrose, as opposed to when harnessed in the lab experiment. This demonstrates that nutrient assessment varies with experimental context (Mommaerts et al., 2013).

As another example, in a learning experiment, harnessed bumblebees could detect and discriminate various amino acids in their food. Ruedenauer et al. submitted harnessed bumblebees to a discriminative learning experiment to test if they were able to perceive various amino acids. They touched the antennae with an amino-acid solution or a water solution and paired one of these solutions with a sucrose reward. In these conditions bumblebees were able to detect amino-acids such as asparagine, cysteine, hydroxyproline, glutamic acid, lysine, phenylalanine, and serine, but not all as alanine, leucine, proline and valine could not be sensed (Ruedenauer et al., 2016).

Nevertheless, they show a preference for high-sugar diets when given a choice between various artificial food sources (Stabler et al., 2015). This highlights that bumblebees will have a higher probability to collect carbohydrates over lipids and proteins, but whether this trend is similar between all individuals, and for which proportions it is observed, is still not clear. This question will be addressed in part A (p.38-44).

Bees of different ages and castes have different nutritional needs

Nutritional needs may vary across a lifetime, and whether bumblebees change their behavioural responses to food at different ages is unknown, contrary to honeybees. Honeybees accomplish different behavioural tasks during their adult lifetime, starting from nursing and nest maintenance just after the emergence, to guarding and foraging after a few weeks (Robinson, 1992). These behavioural transitions are accompanied with various metabolic and physiological changes (Calderone et al., 1989; Toth and Robinson, 2005), where juvenile hormone titers increase and those of vitellogenin

decrease as honeybees start to forage outside the hive, resulting in a higher gustatory sensitivity (Page et al., 1998; Amdam and Rueppell, 2006). Brain expression levels of gustatory receptors also increase between newly emerged bees and foragers (Simcock et al., 2017). As a result of such changes, honeybee foragers focus on carbohydrates intake (Paoli et al., 2014a) while younger workers consume more proteins and lipids via pollen mainly to produce glandular secretions (Crailsheim et al., 1992). Nutritional experiments demonstrate this diet transition, with newly emerged bees that change from an amino-acids:carbohydrates ratio of 1:50 to one of 1:75, while foragers choose a ratio 1:250 (Paoli et al., 2014a).

Whether bumblebees also undergo age-dependent metabolic and physiological changes is unknown. Since their division of labour is not age-based (Goulson, 2010), they may well maintain their food choices irrespective of age, despite their phylogenetic closeness to honeybees, division of labour among bumblebee workers does not correlate with age (Goulson, 2010). This question will be addressed in part B (p.45-52).

Foragers tend to specialize on some resources

Foraging does not simply imply collecting the first food resources encountered, but rather making decisions about collecting from particular type of flowers rather than from others. Because search and flight activities require high energetic input over time (Heinrich, 1979), foragers need to trade-off between minimizing flight distance and prioritizing high reward intake (Lihoreau et al., 2011). When food is patchily distributed in space and/or time, collective foraging becomes an efficient strategy (Lihoreau et al., 2015; 2017), and resource partitioning among workers could increase the scale of a foraging area for a colony (Giraldeau and Caraco, 2018; Dubois et al., 2021). This is illustrated by the specialization of different foragers in collecting nectar or pollen in bumblebees (O'Donnell et al., 2000; Raine and Chittka, 2007) and in honeybees (De Brito et al., 2010).

By monitoring visits of *B. impatiens* foragers on either sucrose solution or pollen dishes, Hagbery and Nieh (2012) found that 16-36% of the foragers were specialists, i.e., collected only one food resource more than 90% of the time. The choice of visited

sources (only nectar, only pollen or both) was made on the first day of foraging, and maintained for several days (Hagbery and Nieh, 2012). A similar result was found on *B. bifarius nearcticus* with 30-40% of foragers focusing on either nectar or pollen collection (O'Donnell et al., 2000). Such behavioural specialization could be a response to social and environmental variations, e.g., changes in colony food stores or available flowers (Plowright et al., 1993; Molet et al., 2008; Francis et al., 2016) or, as in honeybees, the result of inter-individual variability in nutrient perception (Page et al., 1998) and/or genetic background (Calderone et al., 1989; Ben-Shahar et al., 2002).

Whether bumblebee foragers divide their foraging task on a finer nutritional level by specializing on specific food sources based on nutrient ratios is unknown. For example, some foragers might show a preference for nectars richer in proteins while other might focus on carbohydrate-rich ones. This question will be addressed in part C (p.53-59).

Aim of chapter

In this chapter, we asked what drives food choices in individual bees, from the perception of nutrients in food, to the balancing of diet, and how this varies across different ages, castes and social contexts.

In part A, we tested how bumblebees respond to carbohydrates, lipids and proteins. First, bumblebees performed a sensitivity test on each nutrient, and we expected a higher response rate to low concentrations of carbohydrates, compared to other nutrients. Second, we tested if lipids or proteins had an influence on the response to carbohydrates and hypothesized a negative impact.

In part B, we tested how bumblebees balance nutrient collection through age and caste. We gave adults a choice between diets varying in nutritional contents. We expected a different nutritional intake between queens and workers, but not between workers of various ages.

In part C, we observed whether bumblebees specialize in the collection of different nutrients when foraging. Free-flying foragers had a choice between artificial flowers providing diets varying in their nutritional balance. We expected to see foragers visit multiple flowers and balancing their collection of the different nutrients.

A. Perception of nutrients

We investigated bumblebees' responsiveness to carbohydrates, lipids and proteins in two experiments (Mommaerts et al., 2013). First, we measured at which concentration of nutrients bumblebees started to show motivation to collect each nutrient. As bumblebees demonstrated a preference for high-sugar diets over protein (Stabler et al., 2015), we hypothesized they would have a higher tendency to respond to low concentrations of carbohydrates, as compared to the other nutrients. Second, we tested if lipids or proteins have an influence on carbohydrates response. At certain doses, proteins and lipids are toxic to bees (Paoli et al., 2014b; Vaudo et al., 2016b; Ruedenauer et al., 2020). We therefore hypothesized proteins and lipids would negatively affect the motivation to collect solutions rich in carbohydrates when incorporated in them.

Material and Methods

Bumblebees

Experiments were conducted between January and April 2021. Eleven commercial colonies of *Bombus terrestris* (Koppert BV, Berkel en Rodenrijs, Netherlands) were used. Each colony contained about 100 workers, brood cells and a queen. Colonies were transferred to rearing boxes (17x16cm) maintained at 28°C and 50% relative humidity, under a 12:12h light:dark photocycle. Each box was divided in two parts: one for the brood, and one for the food. Colonies were fed ad libitum with 40% (w/w) sugar solution and commercial pollen (ICKO Apiculture, Bollène, France) previously passed through UV light for 4h to kill any potential germ.

Artificial diets

In experiment 1, bumblebees were tested with solutions made of either proteins, lipids, or carbohydrates at increasing concentrations (0.1%; 0.3%; 1%; 3%; 10% w/w). Nutrients

were not tested at concentrations exceeding 10% (w/w) because lipids and proteins would precipitate in water.

In experiment 2, bumblebees were tested on a 30% (w/w) carbohydrate solution, alone or supplemented with increasing concentrations (0.1%; 0.3%; 1%; 3%; 10%) of either lipids or proteins.

In both experiments, carbohydrates consisted of main sugars found in nectar (Nicolson and Thornburg, 2007; Nicolson, 2011), sucrose (Interdis, Massy, France), fructose and glucose (Louis François, Paris, France).

Lipids consisted of sunflower oil (Auchan, Villeneuve d'Ascq, France), coconut oil (Cauvin, Saint-Gilles, France), flaxseed oil (Mon droguiste, Troyes, France), palm oil (Mon droguiste, Troyes, France) and soy lecithin (Louis François, Paris, France).

Proteins consisted of soy protein isolate and brown rice protein (MyProtein, Manchester, England), and 10 essential free amino acids (valine, methionine, isoleucine, leucine, threonine, phenylalanine, lysine, histidine, arginine, tryptophan) (Fisher Scientific, Strasbourg, France).

Within each mixture, the ratio between ingredients was calculated to match as possible a mean profile found in natural pollen for essential amino acid (Szcześna, 2006; Di Pasquale et al., 2013; Somme et al., 2015; Moerman et al., 2016) and fatty acids (Manning, 2006; Ruedenauer et al., 2020).

Xanthan and guar gums (Labo&Gato, Bordeaux, France) were used as emulsifiers and thickening agents to facilitate the mixing of proteins and lipids in water. To prepare 1kg of each diet, 1.2g of both gums were put in 300g of water. All other ingredients were then added with the adequate amount of remaining water. For complete recipes see Table 2.

Table 2: Recipes of the nutrient solutions used in nutritional perception experiments. Quantities of each ingredient in either carbohydrates, lipids or proteins solution at various concentrations. Solutions were complemented with 1.2g of both xanthan and guar gums (Labo&Gato, Bordeaux, France), and water to achieve a kilogram. Differences between mixes and concentration were due to non-nutritional additives in ingredients.

Concentrations	0.1%	0.3%	1%	3%	10%	30%
Carbohydrate Mix (g/kg)	0.10	0.30	1.01	3.03	10.12	30.35
<i>Sucrose</i>	0.05	0.15	0.51	1.52	5.06	15.17
<i>Fructose</i>	0.03	0.09	0.30	0.91	3.03	9.10
<i>Glucose</i>	0.02	0.06	0.20	0.61	2.02	6.07
Lipid Mix (g/kg)	0.10	0.30	1.01	3.04	10.12	
<i>Sunflower</i>	0.01	0.02	0.08	0.23	0.78	
<i>Coconut</i>	0.01	0.02	0.08	0.23	0.78	
<i>Flaxseed</i>	0.03	0.09	0.31	0.93	3.11	
<i>Palm</i>	0.03	0.09	0.31	0.93	3.11	
<i>Soy lecithin</i>	0.02	0.07	0.23	0.70	2.33	
Protein Mix (g/kg)	0.12	0.35	1.17	3.51	11.72	
<i>Soy</i>	0.04	0.12	0.40	1.20	3.99	
<i>Rice</i>	0.06	0.18	0.60	1.80	5.98	
<i>Valine</i>	0.0015	0.0044	0.0146	0.0439	0.1463	
<i>Methionine</i>	0.0007	0.0020	0.0067	0.0200	0.0665	
<i>Isoleucine</i>	0.0016	0.0048	0.0160	0.0479	0.1596	
<i>Leucine</i>	0.0016	0.0048	0.0160	0.0479	0.1596	
<i>Threonine</i>	0.0023	0.0068	0.0226	0.0678	0.2261	
<i>Phenylalanine</i>	0.0004	0.0012	0.0040	0.0120	0.0399	
<i>Lysine</i>	0.0049	0.0148	0.0492	0.1476	0.4920	
<i>Histidine</i>	0.0017	0.0052	0.0173	0.0519	0.1729	
<i>Arginine</i>	0.0001	0.0004	0.0013	0.0040	0.0133	
<i>Tryptophan</i>	0.0027	0.0080	0.0266	0.0798	0.2660	

Behavioural assays

For one test session, 20 bumblebees from the same colony were transferred in a plastic box (17x23cm) 24h before the test. Boxes were placed in an incubator in the dark, at 26-29°C and 70-80% relative humidity. Bees were provided with two drilled 15ml-Eppendorf vials containing each 4ml of 15% (w/w) sugar solution. Food collection was controlled by weighing the vials before the introduction and after the removal of the bees. After 24h, bees were removed and cold-anesthetized for 7-10 minutes and individually mounted in drilled capsules limiting lateral movements but allowing them to stick out their heads (Figure 5), then put back to rest in the incubator for 3h. Subsequently, bees were given water to drink.

Individual responses were evaluated by assessing the reflex proboscis extension response (PER) after touching each bee's antennae for 30 seconds with a toothpick previously soaked in one of the liquid solutions (Mommaerts et al., 2013). Each bee was given successive presentations of one macronutrient at increasing concentrations. Between two successive presentations, bees were presented water to prevent sensitization to the toothpick. The response to each presentation was scored as "+1" for a PER to the nutrient solution; "0" for no extension; "-1" for a PER to water. If the bee stayed in the back of the capsule and could thus not be tested, or was found dead, it was discarded. The score could reach +5, if bumblebees responded to all diet concentrations and never to water, and -5 if they only responded to water and never to the diets. In total, we tested 667 bumblebees. 36 were found dead during the test and thus discarded from the analysis.

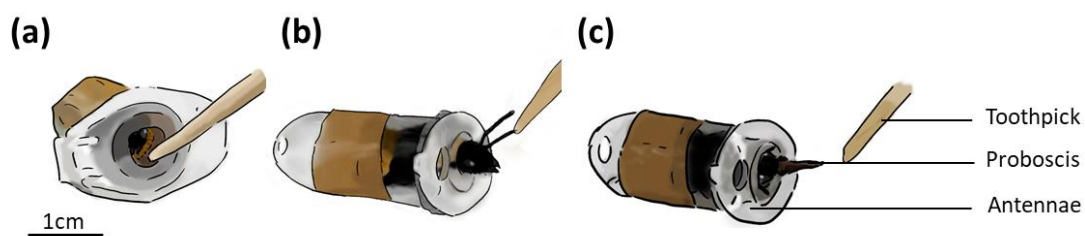


Figure 5: Nutrient sensitivity test capsule with bumblebee. Bumblebees were mounted in drilled capsules limiting lateral movements but allowing them to stick out their heads. Individual responses were evaluated by assessing the reflex proboscis extension response (PER) after touching each bee's antennae with a toothpick previously soaked in solution. **(a)** Bee that stayed at the bottom of the tube. **(b)** Bee's antennae touched with soaked toothpick. **(c)** Proboscis extension that demonstrate bee's motivation to collect what was presented.

Body size

As body size affects foraging efficiency and task repartition in bumblebees (Goulson et al., 2002), we controlled if it had an influence on responsiveness of nutrients. After the behavioural tests, all bees were frozen in order to measure their thorax width (intertegular span), as a proxy of body size, using a binocular microscope (Nikon Europe B.V., Amsterdam, Netherland). Bees were then dried at 80°C during 4h in an oven (WTB Binder, Tuttlingen, Germany) before being weighed using a precision balance of 1mg (ME103T, Mettler Toledo, Greifensee, Switzerland).

Statistical analyses

Data are presented in the text as median with the limits of 95% confidence intervals into brackets. All statistical analyses were performed in R 4.1.2 (R Core Team, 2021). To test the difference of proportions of responses to different group stimuli (nutrient in solution) and concentrations we used a Chi-Square Test of Independence (function *chisq.test* in package “stats”). A body size effect on response scores was tested using an ANOVA (function *anova* in package “stats”) from a linear regression model (function *lme* in package “stats”). Multiple comparisons were performed with group-by-group 95% confidence intervals difference of medians (generated by bootstrapping).

Results

Experiment 1: Nutrient responses

Bumblebees responded more to carbohydrates than to lipids or proteins

A total of 128 bees per group stimulus were tested in experiment 1. Proportions of PER responses differed with increasing concentrations of carbohydrates ($\chi^2_{(4,N=5)} = 63.64$, $p < 0.001$), so that more responses were observed at 1%, 3% and 10% respectively (Figure 6.a). However, no such effect was observed in bees tested with lipids ($\chi^2_{(4,N=5)} = 1.77$, $p = 0.78$) or proteins ($\chi^2_{(4,N=5)} = 1.26$, $p = 0.87$) (Figure 6.a). In total, 53 out of 128 bees responded to at least one concentration of the carbohydrate solution, while only 23 and 13 responded to the lipid and protein solutions, respectively.

Adult body size did not influence nutrient responses

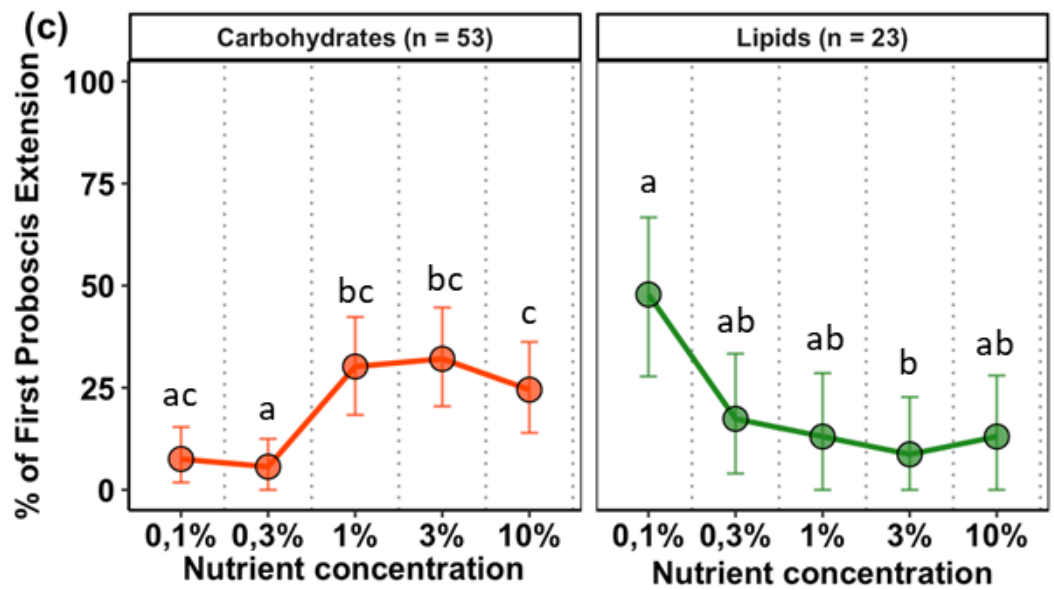
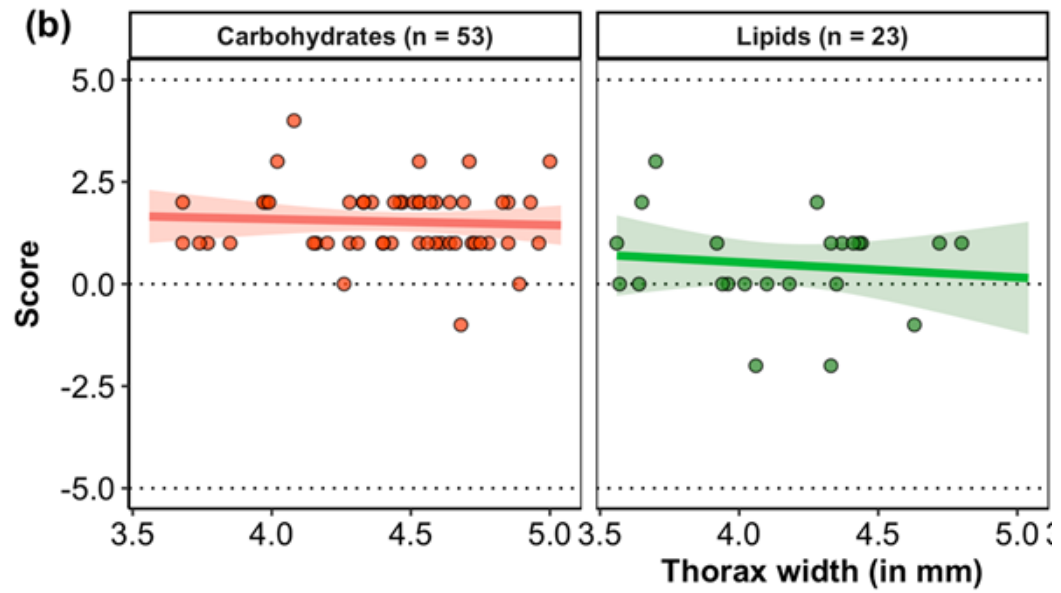
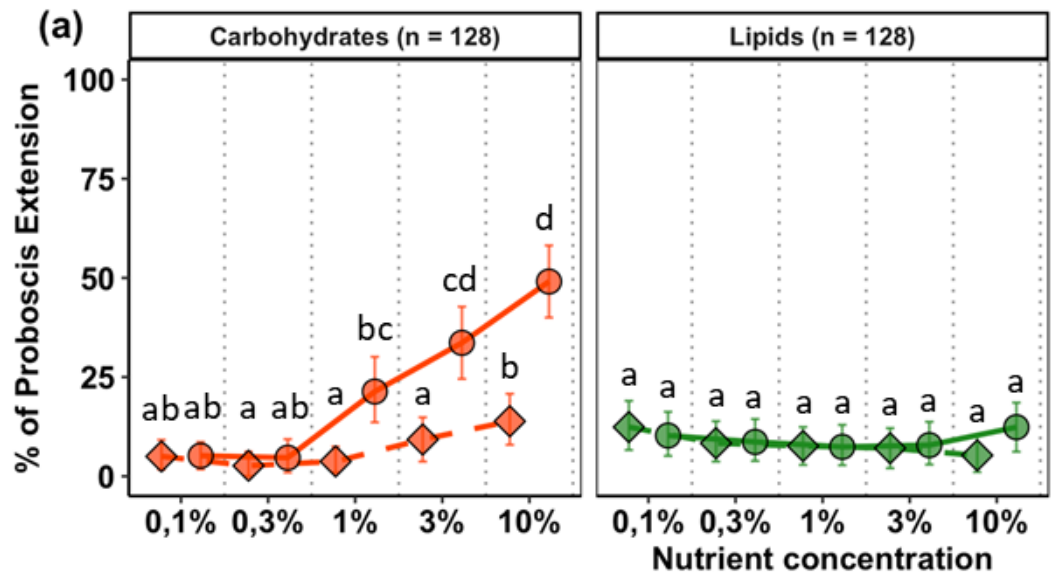
Bees obtained average scores of 1.53 [1;2.1] when tested with carbohydrates, 0.48 [-0.3;1.3] with lipids and 1.15 [0.5;1.9] with proteins (Figure 6.b). There was no significant difference between groups after pairwise comparison tests (carbohydrates x lipids: 1.05 [-0.3;2.5]; carbohydrates x proteins: 0.37 [-0.9;1.6]; lipids x proteins: -0.68 [-2.3;0.8]). We did not observe any effect of body size (thorax width) on their individual scores ($F_{(1,87)} = 0.32$, $p = 0.57$, $R^2 = -0.01$).

Bees were motivated to lower concentrations of lipids compared to carbohydrates

Proportions of first responses to the solution differed across concentrations for carbohydrates ($\chi^2_{(4,N=5)} = 31.54$, $p < 0.001$), lipids ($\chi^2_{(4,N=5)} = 50.28$, $p < 0.001$) and proteins ($\chi^2_{(4,N=5)} = 21.3$, $p < 0.001$) (Figure 6.c). Most of the bees began to respond to carbohydrates at 1%, 3% or 10%, but to lipids already at 0.1%. No differences were found with pairwise comparisons for bees tested with proteins solution, certainly due to the small number that responded to proteins ($N = 13/128$).

Conclusion

We found that bumblebees respond differently to carbohydrates, lipids and proteins. More individuals responded to carbohydrates than to lipids and proteins respectively. However, when responding, they started doing so at lower concentrations for lipids than for carbohydrates. Finally, we did not observe neither a body size effect on the nutrient responses, nor a positive or negative influence of lipids and proteins on carbohydrates responses.



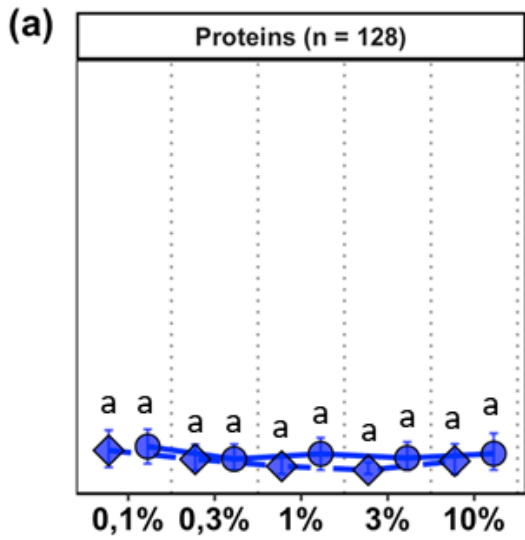
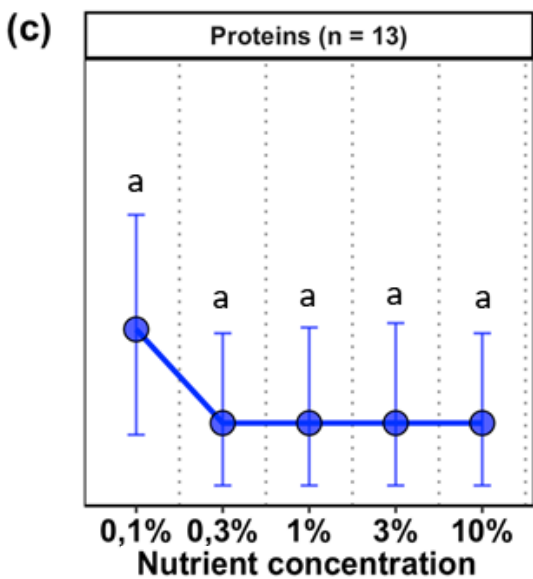
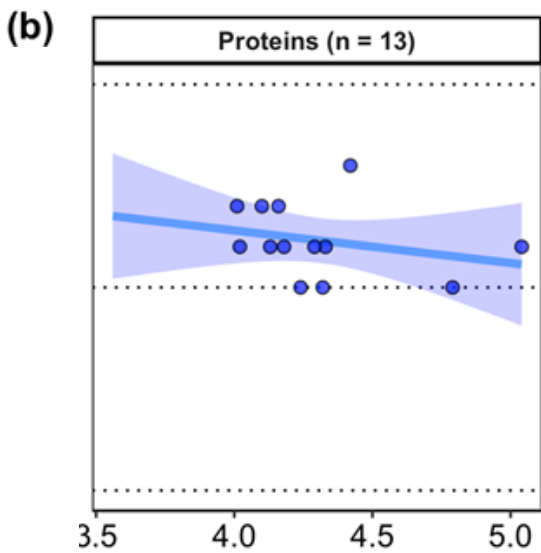
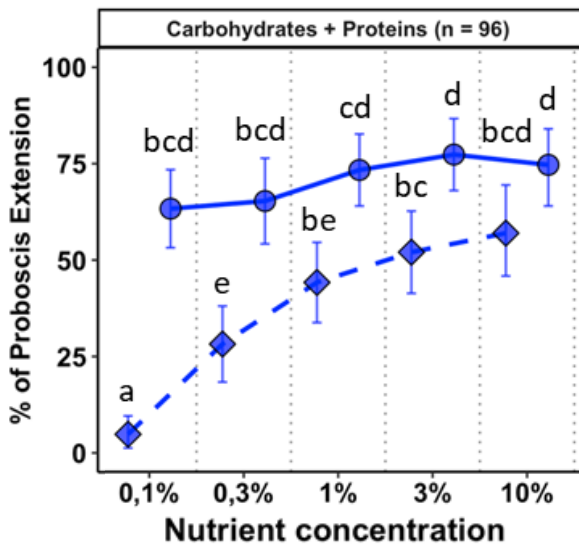
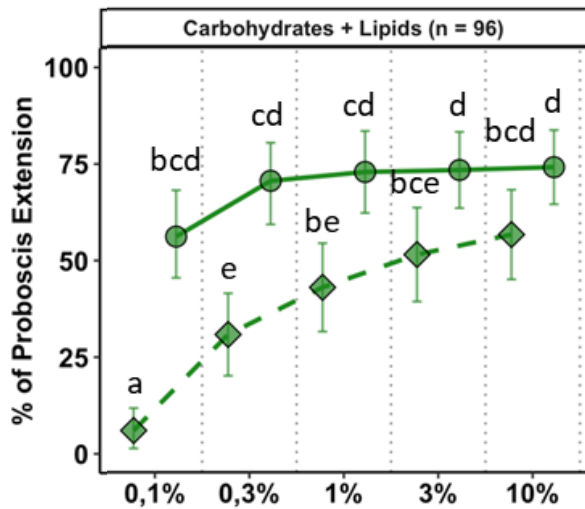
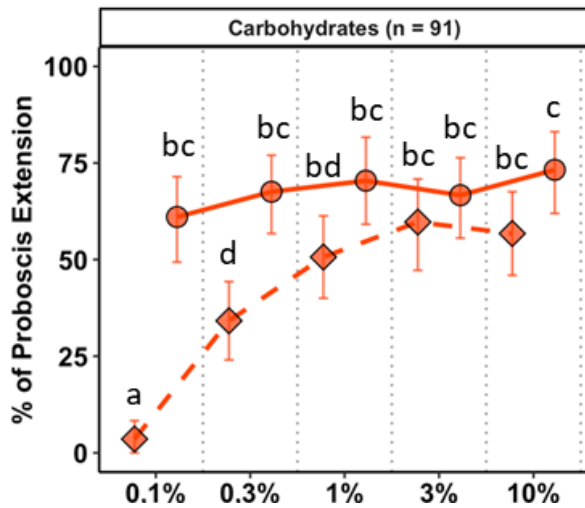


Figure 6: Bee responses to increasing concentrations of carbohydrates, lipids or proteins. (a) Mean proportions of responses (proboscis extension) to each concentration, for each solution (solid lines, circle dot) and to water presentations (dashed lines, diamond dot). Bars represent confidence intervals at 95% as generated via bootstraps. (b) Mean individual scores of bees that responded to at least one concentration, plotted over their thorax width (intertegular span), as a proxy of body size. Linear regressions and their confidence interval are represented by colored lines. (c) Mean proportions of the first responses made by bumblebees that responded to at least one concentration. Bars represent confidence intervals at 95% generated via bootstraps. Letters stand for significant difference after pairwise comparison tests in each stimulus group.



Stimulus — Carbohydrates — Lipids — Proteins
Test ◇ Water ○ Food



Stimulus — Carbohydrates — Carbohydrates + Lipids — Carbohydrates + Proteins
 Test ◊ Water ○ Food

Figure 7: Influence of lipids and proteins on carbohydrates responses by bumblebees. Mean proportions of responses (proboscis extension) made by bumblebees tested on carbohydrate-only solutions, or on 30% (w/w) carbohydrates added with increasing concentrations of either lipids or proteins. Solid lines and circle dots represent responses to the liquid solution and dashed lines and diamond dots, those to water. Bars represent confidence intervals at 95% generated via bootstraps. Letters stand for significant difference after pairwise comparison tests in each stimulus group.

B. Regulation of nutrient collection

As we observed that bees perceive and react differently to the three macronutrients, we then tested how they balance their nutrient collection through age and across castes when given the opportunity to select among different food types. Individual queens, and workers aged either 0-7, 8-14 or 15-21 days, had the choice between four artificial diets varying in their contents in carbohydrates, lipids and proteins, during three days in the laboratory. We hypothesized a different nutritional intake between queens and workers, but not between workers of various ages like in honeybees (Paoli et al., 2014a), since division of labour among bumblebee workers does not correlate with age (Goulson, 2010).

Material and Methods

Bumblebees

Experiments were conducted between March and May 2021. A total of 16 commercial colonies of *B. terrestris* (Koppert BV, Berkel en Rodenrijs, Netherlands) were used. Each colony contained about 50 workers, brood cells and a queen. Upon reception of the colonies, all workers were removed. After one week, newly emerged workers were removed from the colony and placed in a box (17x16cm) to form microcolonies. This operation was repeated to obtain three groups of workers of controlled age (0-7 days; 8-14 days; 15-21 days). Colonies and micro-colonies were maintained at 28°C and 50% relative humidity, under a 12:12h light:dark photocycle. Room temperature was controlled every five minutes using a data-logger (Elitech, London, United Kingdom). Colonies and micro-colonies were fed *ad libitum* with commercial sugar syrup (Koppert BV, Berkel en Rodenrijs, Netherlands) and commercial pollen (ICKO Apiculture, Bollène, France) previously passed through UV light for 4h to kill any potential germ.

Artificial diets

We designed fully artificial liquid diets made of proteins, lipids, carbohydrates, and vitamins. For the sake of simplicity, we did not use solid diets that could be used for nest building and for which measures of food collection would be less precise. Our 12 diets varied in their ratios of proteins, lipids, and carbohydrates (Table 3 and Figure 10), but were isocaloric (0.63 cal/mg). This calculation was based on workers' daily consumption provided from previous studies (about 190 calories for 300 mg of diet per day: Stabler et al., 2015; Vaudo et al., 2016b), and considering that proteins and carbohydrates provide about 4 cal/mg and lipids about 9 cal/mg (Lii et al., 1975).

Diets were composed of the same mix of carbohydrates, lipids and proteins as in previously (Chapter 1.A, page 39), with the addition of 5g of vitamins per kilogram (Vanderzant vitamins mix, Sigma-Aldrich, Darmstadt, Germany). Xanthan and guar gums (Labo&Gato, Bordeaux, France) were used as emulsifiers and thickening agents to facilitate the mixing of proteins and lipids in solution. The water content of each diet was adjusted in order to complement the weight of nutrients up to 1kg (Table 3). Each diet was mixed using a blender before storage in the freezer. All diets had nutrient concentrations (proteins: 1.5-48.4%; lipids: 1.2-24.6%; carbohydrates: 10-70%) falling within the range of natural pollens and nectars (Roulston and Cane, 2000; Weiner et al., 2010; Nicolson, 2011; Vaudo et al., 2020). Preliminary observations showed that bumblebees collected all diets and stored them in empty cells (i.e., honey pots) (Figure 8). Over the manuscript, we used "diet" to refer as a nutrient ratio achieved or used, and "food" as a collected quantity.

Table 3: Recipes of the 12 artificial diets used in regulation of nutrient experiment. Percentages of carbohydrates, lipids, proteins in relation to total nutrients in calories. Amounts of carbohydrates, lipids, proteins, total nutrients, and water in grams per kilograms. Ratio of the three nutrients in relation to the two others in calories, and ratio of all nutrients in relation to water. Nutrients mixed were composed of the same ingredient in similar proportions as described in Chapter 1.A (see Methods page 39). Differences between mixes and concentration were due to non-nutritional additive in ingredients.

Diets	1	2	3	4	6	14
% Carbohydrate	90.00	75.83	75.83	61.66	61.66	33.33
% Lipid	5.00	5.00	19.16	5.00	33.33	47.50
% Protein	5.00	19.16	5.00	33.33	5.00	19.16
C in diet (g/kg)	142.50	120.06	120.06	97.63	97.63	52.77
L in diet (g/kg)	3.51	3.51	13.48	3.51	23.45	33.42
P in diet (g/kg)	7.91	30.34	7.91	52.77	7.91	30.34
Total Nutrient (g/kg)	153.93	153.93	141.47	153.93	129.01	116.55
Water (g/kg)	835.60	832.02	848.21	828.43	860.81	869.83
Ratio N : W	5.43 : 1	5.41 : 1	6 : 1	5.38 : 1	6.67 : 1	7.46 : 1
Ratio C : (L+P)	9 : 1	3.14 : 1	3.14 : 1	1.61 : 1	1.61 : 1	1 : 2
Ratio L : (C+P)	1 : 19	1 : 19	1 : 4.22	1 : 19	1 : 2	1 : 1.11
Ratio P : (C+L)	1 : 19	1 : 4.22	1 : 19	1 : 2	1 : 19	1 : 4.22
Carbohydrate Mix (g/kg)	144.15	121.46	121.46	98.77	98.77	53.39
Lipid Mix (g/kg)	3.55	3.55	13.64	3.55	23.73	33.81
Protein Mix (g/kg)	9.27	35.55	9.27	61.83	9.27	35.55

Diets	15	16	22	23	27	28
% Carbohydrate	33.33	19.16	5.00	5.00	5.00	5.00
% Lipid	61.66	5.00	5.00	19.16	75.83	90.00
% Protein	5.00	75.83	90.00	75.83	19.16	5.00
C in diet (g/kg)	52.77	30.34	7.91	7.91	7.91	7.91
L in diet (g/kg)	43.39	3.51	3.51	13.48	53.36	63.33
P in diet (g/kg)	7.91	120.06	142.5	120.06	30.34	7.91
Total Nutrient (g/kg)	104.08	153.93	153.93	141.47	91.62	79.16
Water (g/kg)	886.03	817.67	814.08	830.27	895.05	911.24
Ratio N : W	8.51 : 1	5.31 : 1	5.29 : 1	5.87 : 1	9.77 : 1	11.51 : 1
Ratio C : (L+P)	1 : 2	1 : 4.22	1 : 19	1 : 19	1 : 19	1 : 19
Ratio L : (C+P)	1.61 : 1	1 : 19	1 : 19	1 : 4.22	3.14 : 1	9 : 1
Ratio P : (C+L)	1 : 19	3.14 : 1	9 : 1	3.14 : 1	1 : 4.22	1 : 19
Carbohydrate Mix (g/kg)	53.39	30.70	8.00	8.00	8.00	8.00
Lipid Mix (g/kg)	43.90	3.55	3.55	13.64	53.98	64.07
Protein Mix (g/kg)	9.27	140.66	166.94	140.66	35.55	9.27

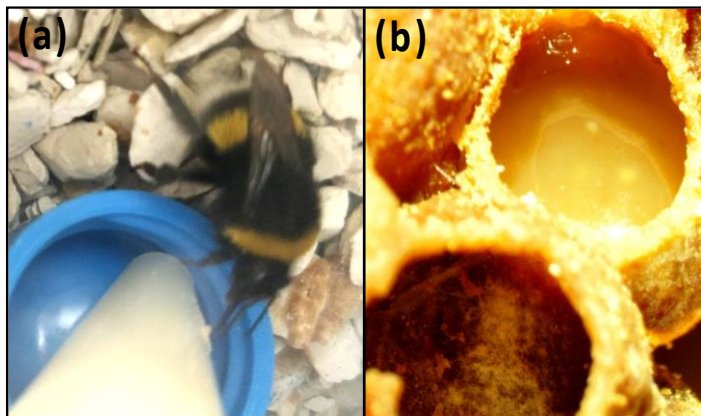


Figure 8: Collection of artificial diet by bumblebees. The artificial diets were provided in gravity feeders (drilled Eppendorf of 5ml), from which they were collected by bumblebees for (a) immediate consumption or (b) storage in empty cells.

For each configuration, bumblebees were provided four drilled 5ml-Eppendorf of in their peripheral chamber, containing 1.26g (± 0.01 CI95) of each diet. All diets were renewed every day with a new cup to collect leakage; each vial and cup were weighed before and after being provided to the bees, using a precision balance of 1mg (ME103T, Mettler Toledo, Greifensee, Suisse). Precise time in hour between each renewal was measured. The amount of leaking diet was measured by putting vials in an empty chamber, following the same procedure, and was subtracted from the measured diet collection (see calculation below). Dead bees were removed and frozen every day.

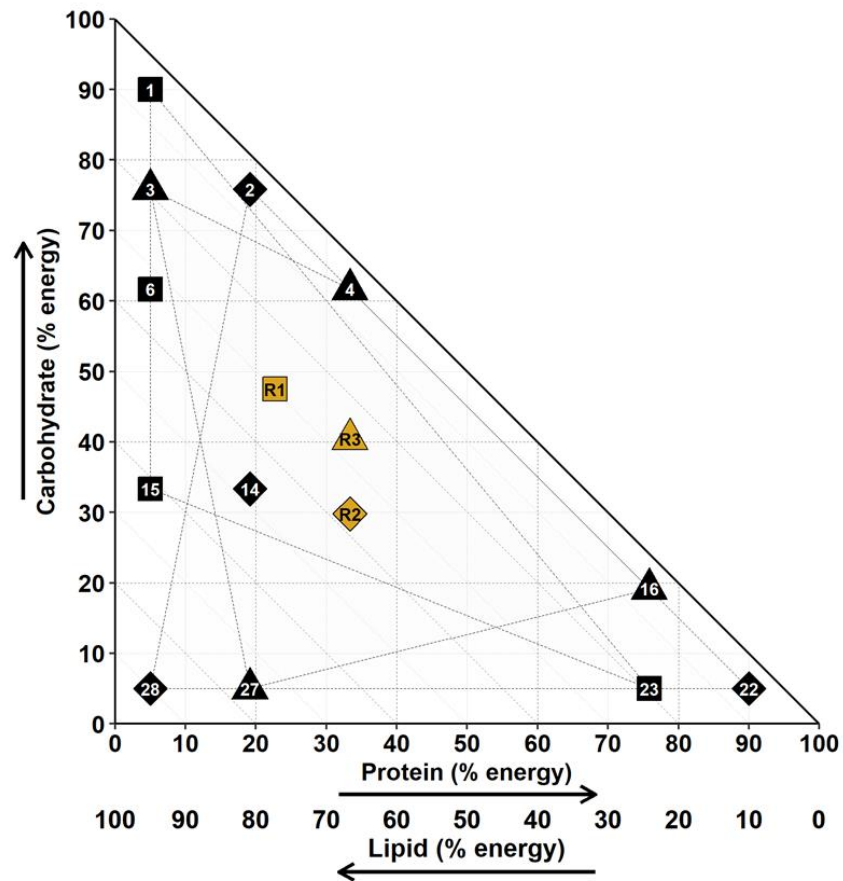


Figure 10: Distribution of the 12 artificial diets in a 3D nutrient space for choice experiments. Each numbered symbol represents a specific diet defined by its proportions of carbohydrates, lipids and proteins. Symbol shapes (squares, diamonds or triangles) correspond to three different choice configurations (each with four diets). For each configuration (1-3), the yellow symbol represents the proportions of collected micronutrients expected under the assumption of random (R) nutrient collection.

Measure of food collection

We measured the average amounts of total food and of each specific nutrient collected per bee each day as follows.

Initial and remaining amounts of food:

$$\text{Initial Food} = \text{Food weight before Test} - \text{Median Tube weight}$$

$$\text{Remaining Food} = \text{Food weight after Test} - \text{Median Tube weight} + \text{Cup weight after Test} - \text{Median Cup weight}$$

Measure of leakage:

$$\text{Leakage (\% per hour)} = \frac{(\text{Initial Food} - \text{Remaining Food})}{\text{Initial Food} * \text{Time}} * 100$$

Amount of collected food:

$$\text{Daily Collected Food per Bee} = \text{Initial Food} * (1 - \text{Median Leakage} * \text{Time}/100) - \text{Remaining Diet}$$

$$\text{Cumulative Collected Food per Bee} = \sum_{i=0}^{\text{Day}} \text{Daily Collected Food per Bee at Day } i$$

Amounts of each nutrient collected:

$$\text{Collected Nutrient per Bee} = \text{Cumulative Collected Food per Bee} * \% \text{ of Nutrient in Food}$$

Note that the percentages of nutrient in food could be based on calories or milligrams.

Body size

We measured the body size and weight of every bee to control for potential size effects using thorax width and dry weight as described in Chapter 1.A. (see Methods page 41).

Statistical analysis

Data are presented in the text as median with the limits of 95% confidence intervals into brackets. All statistical analyses were performed in R 4.1.2 (R Core Team, 2021). To test the distribution between groups, we used a Chi-Square Test of Independence (function *chisq.test* in package “stats”). Differences in total diet and nutrient collection were tested with a Kruskal-Wallis test (function *kruskal.test* in package “stats”). Body size effect on total diet collection was tested using an ANOVA (function *anova* in package “stats”) from a linear regression model (function *lme* in package “stats”). Multiple comparisons were performed with group-by-group 95 confidence intervals difference of median (generated by bootstrapping).

Results

Amounts of collected food were not difference across age, caste or body size

Groups of age and caste were not equally distributed. We had more bees of 0-7 days due to higher mortality in bees of 8-14 days and 15-21 days ($\chi^2_{(3,N=292)} = 36.83$, $p < 0.001$). We only had 10 queens because there was only one per colony. Bees which were found dead had collected more (189.93 mg [75.19;354.62]) than those still alive at the end of the experiment (86.63g [27.05;203.83]) (Kruskal-Wallis: $H_{(1)} = 28.19$, $p < 0.001$).

Queens tended to collect more food (187.52g [73.43;290.34]) than isolated workers at all ages (“0-7 days”: 62.49g [25.53;194.54]; “8-14 days”: 91.29g [7.25;217.48]; “15-21 days”: 110.33g [26.51;194.11]), but no significant difference were found (Kruskal-Wallis: $H_{(3)} = 5.56$, $p = 0.14$) (Figure 11.a). Workers’ size did not influence the total amount of food they collected ($F_{(1,88)} = 1.01$, $p = 0.32$) (Figure 11.b).

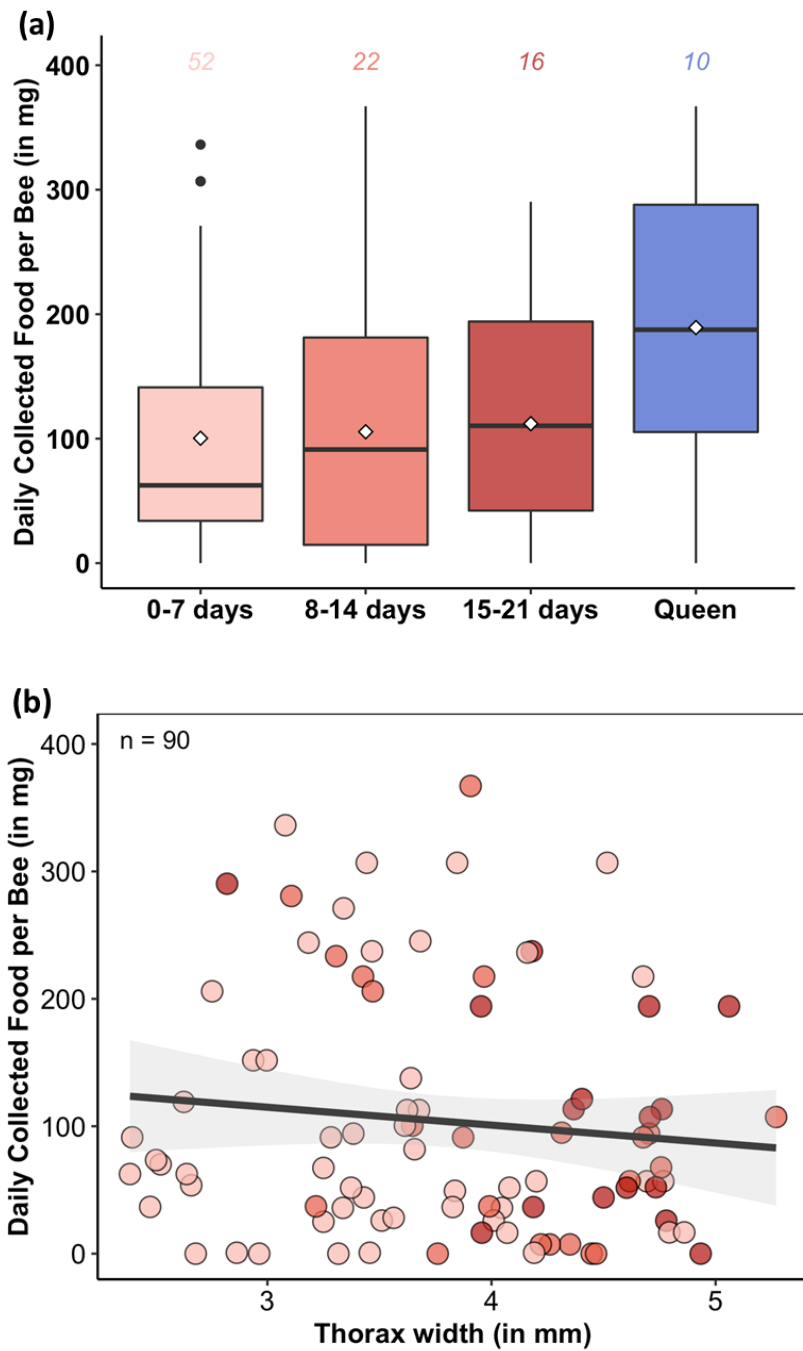


Figure 11: Amounts of total food collected daily by bees of different age and caste. (a) Boxplot of daily collected food (in mg/bee) for each group. The central line is the median and the mean is indicated by a diamond. The edges of the box are the 25th and 75th percentiles, the whiskers extend to the most extreme data points without considering outliers (represented by dots). **(b)** Daily collected food (in mg/bee) plotted over the worker's thorax width (in mm), with the linear regression and its confidence interval.

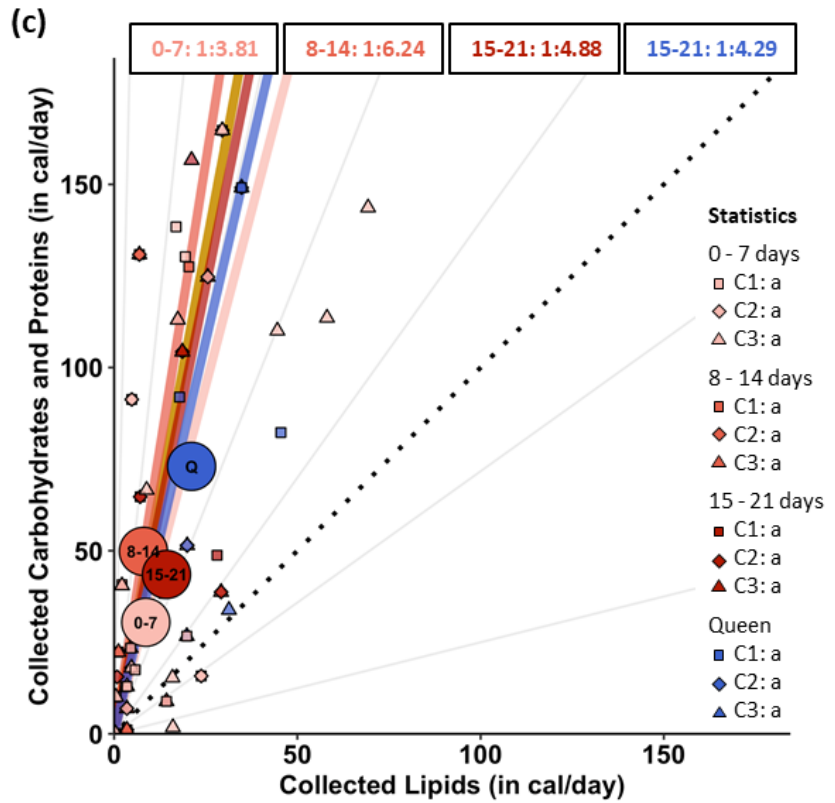
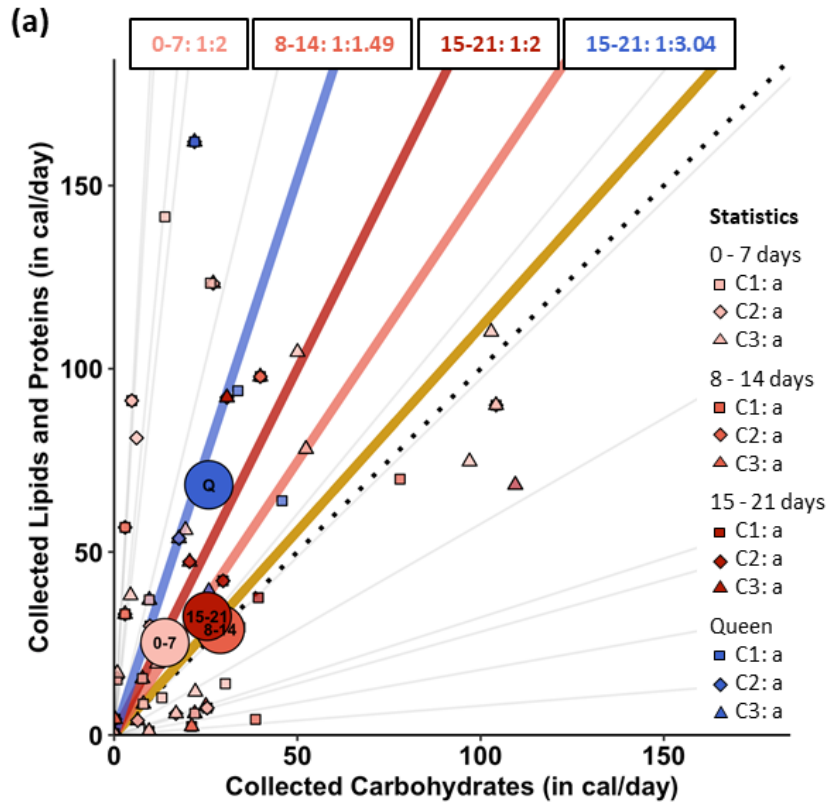
Neither nutrient collection nor nutritional balance was age or caste-dependent

Choice configuration did not have an effect on collected amounts of carbohydrates, lipids or proteins, regardless of age and caste (multiple Kruskal-Wallis: $p > 0.05$). Workers collected similar daily amounts of lipids (0-7 days: 8.64 cal [4.1;19.94]; 8-14 days: 7.99 cal [3.48;17.36]; 15-21 days: 14.29 cal [4.53;28.1]) and proteins (0-7 days: 16.6 cal [1.28;60.55]; 8-14 days: 20.87 cal [1.17;68.11]; 15-21 days: 18.13 cal [1.4;73.54]), while older workers tended to collect more carbohydrates (0-7 days: 13.85 cal [4.8;32.63]; 8-14 days: 28.96 cal [2.96;45.48]; 15-21 days: 25.37 cal [7.72;30.78]). Queens daily collected 25.85 calories of carbohydrates [17.69;71.62], 21.13 of lipids [17.85;40.13] and 47.17 of proteins [17.08;127.89] (Figure 12).

Group age and caste did not influence the ratios between micronutrients: C:(L+P), L:(C+P) and P:(C+L) (Kruskal-Wallis: $H_{(3)} < 1.252$ and $p > 0.05$ in all cases) (Figure 12). Despite inter-individual variability, overall bees seemed to strictly conserve a specific L:(C+P) ratio of 1:4.25 [2;7] as reflected by the linear evolution of this parameter across groups (Figure 12.b).

Conclusion

We found no influence of neither age, body size, nor caste, on total food collection or nutrient balance by bees, although queens tended to collect two times more proteins than workers did, and younger workers (0-7 days) tended to collect less carbohydrates than older ones. In addition, all groups seemed to confine on a unique proportion of lipids in their collected food. However, these conclusions are based on relative low sample sizes.



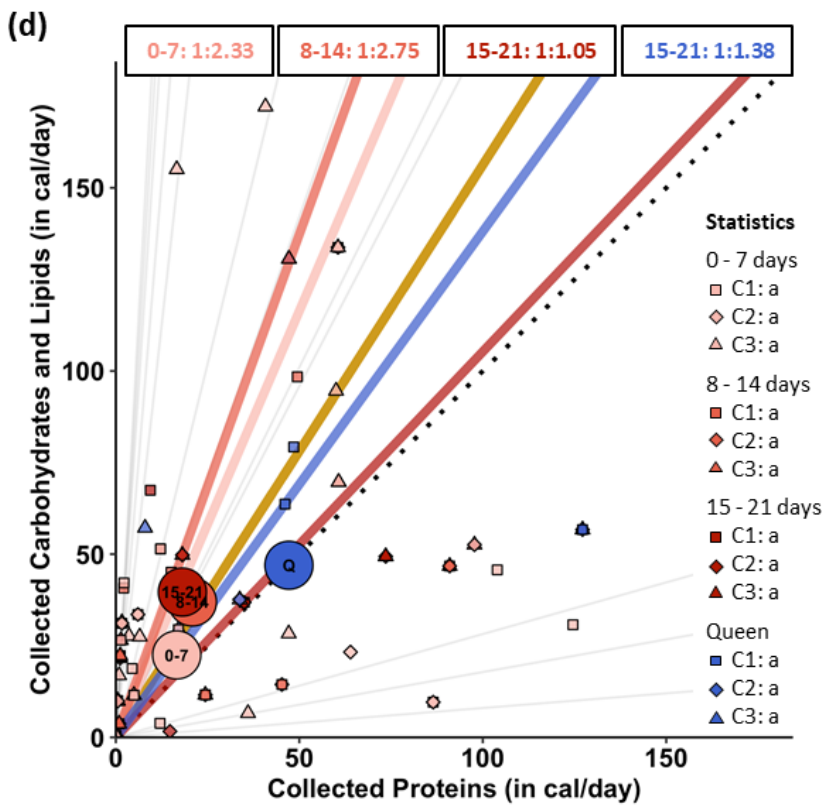
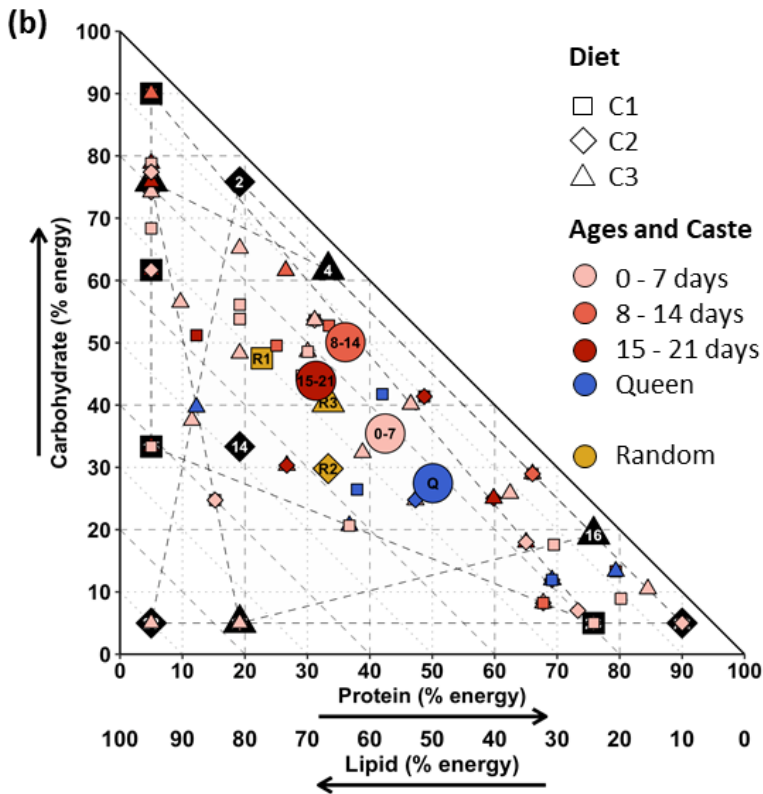


Figure 12: Age and caste effects on collected nutrients.

Nutritional geometric frameworks representing the respective amounts of collected nutrients in two dimensions (a,c,d) and a percentage of collected nutrients in three dimensions (b).

(a) carbohydrates vs. lipids and proteins, (c) lipids vs. carbohydrates and proteins, (d) carbohydrates vs. lipids and proteins.

Grey lines: ratio in each diet; yellow line: ratio expected for a random choice; coloured lines: median ratio obtained for each group; dotted line: 1:1 ratio.

(b) Simultaneous representation of proportions of all nutrients in three dimensions, where the dotted polygons are the three choice configurations with their respective four diets (numbered square, diamond or triangle) and corresponding random points (yellow square, diamond or triangle). In all panels, coloured squares, diamonds or triangles indicate the proportions collected by each individual at the end of the experiment, while coloured circles indicate the median for each group (0-7, 8-14, 15-21, Q). Different letters stand for significant differences.

C. Individual specialization for nutrients

We could not observe any clear difference in the proportions of nutrients collected by workers, therefore we wanted to verify if this was also true in more ecological conditions, i.e., in a free-flying foraging context involving several individuals at a time. Here, we tested if bumblebee foragers could self-organise by specializing individually in collecting different nutrients. We studied their foraging patterns on four types of artificial flowers providing food with varying macronutrient ratios. We hypothesized that foragers would visit multiple flowers and balance between nutrients, as bumblebees demonstrate their ability to make a non-random choice in similar laboratory experiments (Stabler et al., 2015; Vaudo et al., 2016b; Kraus et al., 2019a).

Material and Methods

Bumblebees

Experiments were conducted with two commercial colonies of *B. terrestris* (Koppert BV, Berkel en Rodenrijs, Netherlands), between July and August 2020. Each colony contained about 200 workers, brood cells and a queen. Upon reception, each colony was housed in a wooden nest box (33x17x17cm) with a transparent entrance tube where bumblebees could move in and out of the nest (Figure 13). The entrance tube had five sliding doors to manually control which bumblebee could enter or leave the nest. One colony at a time was placed in the centre of an outdoor flight tent (20x10x4m) (Figure 14.a) and fed diets using artificial flowers from which foragers could freely access (Figure 14.b and c). Each day, 20ml of a sugar solution at 25% (w/w) was given inside the colony.

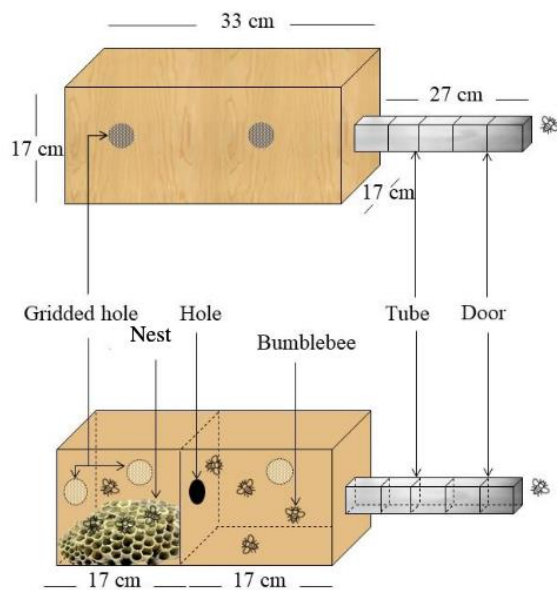


Figure 13: Schematic representation of the nest box housing a bumblebee colony. The nest chamber was opened by three gridded holes and separated by an empty chamber opened by two gridded holes. Bumblebees could freely move from one chamber to the other through a hole. Foragers could leave the colony through a 5-door entrance tube. The doors could be manually controlled to control traffic.

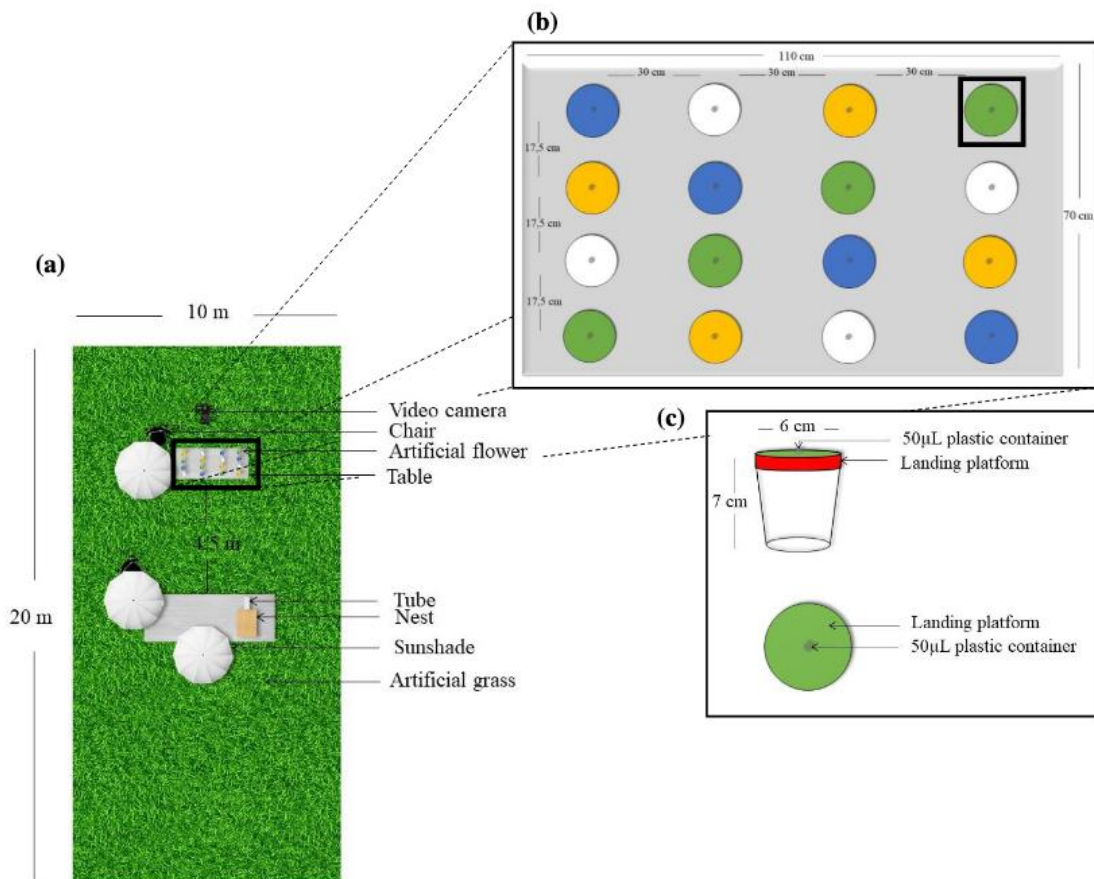


Figure 14: Schematic representation of the free-flying experimental design. (a) Representation of the flight tent during the test. The colony nest box was placed in the centre of the outdoor flight tent. Artificial flowers were placed on a table located 4.5m from the nest. A video camera was positioned on a tripod, next to the table, to record the behaviour of bumblebees. (b) Example of flower array during an experiment: flowers of different colours were placed in a regular array of four lines and four columns on the table, pseudo-randomly with the four colours in each column and line. For each colony, the diet was assigned randomly to one colour. (c) Schematic representation of one artificial flower used during the experiment. Each flower was filled with 30µL of one of the four artificial diets.

Artificial flowers

We used artificial flowers with a circular-coloured plastic disk (diameter: 6cm, height: 7cm) as a landing platform containing the diet in the centre. There were two kinds of flowers with the same shape and size. The training flowers (used for habituating bumblebees to the device) had a landing platform with a hole in the centre to let out a cotton soaked with a sugar solution at 25% (w/w) providing food ad libitum. The test flowers had a central transparent plastic container housing a volume of up to 30 μ L of liquid diet (Figure 14.c). The colours of the landing platform for training flowers were always kept the same (the plastic disk was filled by $\frac{1}{4}$ blue, $\frac{1}{4}$ white, $\frac{1}{4}$ green, $\frac{1}{4}$ yellow), whereas colours for the test flowers was either totally blue, yellow, green, or white (Figure 14.b and c) to facilitate diet discrimination by the bees.

Artificial diets

We designed artificial liquid diets using the approach described in Chapter 1.B (see Methods pages 45-46). We used four diets varying in their ratios of carbohydrates, lipids, and proteins (Table 4 and Figure 15), with the addition of an equal amount of sucrose solution at 50% (w/w).

Table 4: Recipes of the four diets used in the free-flying experiment. Percentages of carbohydrates, lipids, proteins in the four diets in relation to total nutrients in calories. Amounts of carbohydrates, lipid, proteins, total nutrients, sucrose at 50% (w/w) and water in grams per kilograms. Ratio of the three nutrients in relation to the two others in calories, and ratios of all nutrients in relation to water. Nutrients mixed are composed of the same ingredient in similar proportions as described in Chapter 1.A (see Methods page 39). Differences between mixes and concentration were due to non-nutritional additive in ingredients.

Diets	1	2	3	4
% Carbohydrate	90.78	90.78	80.56	80.56
% Lipid	8.02	1.20	1.20	18.24
% Protein	1.20	8.02	18.24	1.20
C in diet (g/kg)	298.82	298.82	265.17	265.17
L in diet (g/kg)	11.73	1.76	1.76	26.68
P in diet (g/kg)	3.96	26.39	60.03	3.96
Total Nutrient (g/kg)	314.51	326.97	326.97	295.81
Water (g/kg)	680.41	664.22	658.84	699.32
Ratio N : W	2.16 : 1	2.03 : 1	2.01 : 1	2.36 : 1
Ratio C : (L+P)	9.85 : 1	9.85 : 1	4.14 : 1	4.14 : 1
Ratio L : (C+P)	1 : 11.47	1 : 82.16	1 : 82.16	1 : 4.48
Ratio P : (C+L)	1 : 82.16	1 : 11.47	1 : 4.48	1 : 82.16
Sucrose 50% (w/w)	250.00	250.00	250.00	250.00
Carbohydrate Mix (g/kg)	49.39	49.39	15.35	15.35
Lipid Mix (g/kg)	11.87	1.78	1.78	26.99
Protein Mix (g/kg)	4.64	30.92	70.33	4.64

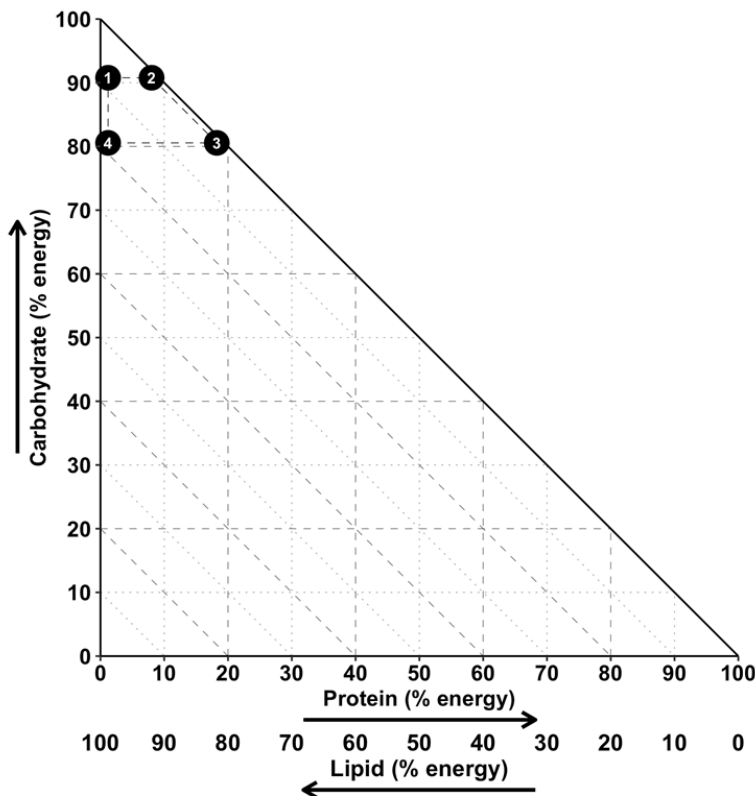


Figure 15: Distribution of the four artificial diets in a 3D nutrient space for free-flying experiment. Each numbered dot represents a specific diet defined by its proportions of carbohydrates, lipids and proteins.

Behavioural assays

On the first day, bumblebees were freely trained to forage on artificial flowers. A training flower was first placed at the colony nest box entrance so that foragers could touch the landing platform and find a 25% (w/w) sugar solution upon leaving the nest. The flower was then gradually moved away from the tube until being placed on a table at a 4.5m distance. This procedure took about six hours and let us tag motivated foragers that regularly foraged on flowers. The test was then conducted during four consecutive days from 8:30am to 12:30am. Several bumblebees could forage at the same time. Every time a new bumblebee visited a flower, it was caught and tagged to allow its identification. Foragers had the choice among 16 test flowers (four colours, one per diet) arranged in random arrays that were changed every day (Figure 14.b). Each flower was filled with 30 μ L of one diet and refilled with the same diet 3 minutes after a forager collected it, and every 30 minutes to keep the diets fresh. We noted the sequence of flowers collected by one bee. The experiment was recorded with a video camera (Sony[®] FDR-AXP33).

Statistical analysis

Data are presented in the text as median with the limits of 95% confidence intervals into brackets. All statistical analyses were performed in R 4.1.2 (R Core Team, 2021). The influence of the number of flower visits on the percentage of collected nutrient was tested using an ANOVA (function *anova* in package “stats”) from a linear regression model (function *lme* in package “stats”). Multiple comparisons were performed with group by group 95 confidence intervals difference of median (generated by bootstrapping).

Results

Bumblebees are nutrient generalists

In total, 56 bumblebees went out to forage, among which 29 visited less than 10 flowers, 20 visited 10 to 30 flowers, and 7 visited more than 30 flowers. Only 8 bees visited a single flower type, and those foragers made less than 10 flower visits in total (Figure 16). Otherwise, most foragers kept visiting multiple types of flowers over the test duration (Figure 17). Bumblebees could thus be considered as nutrient generalists.

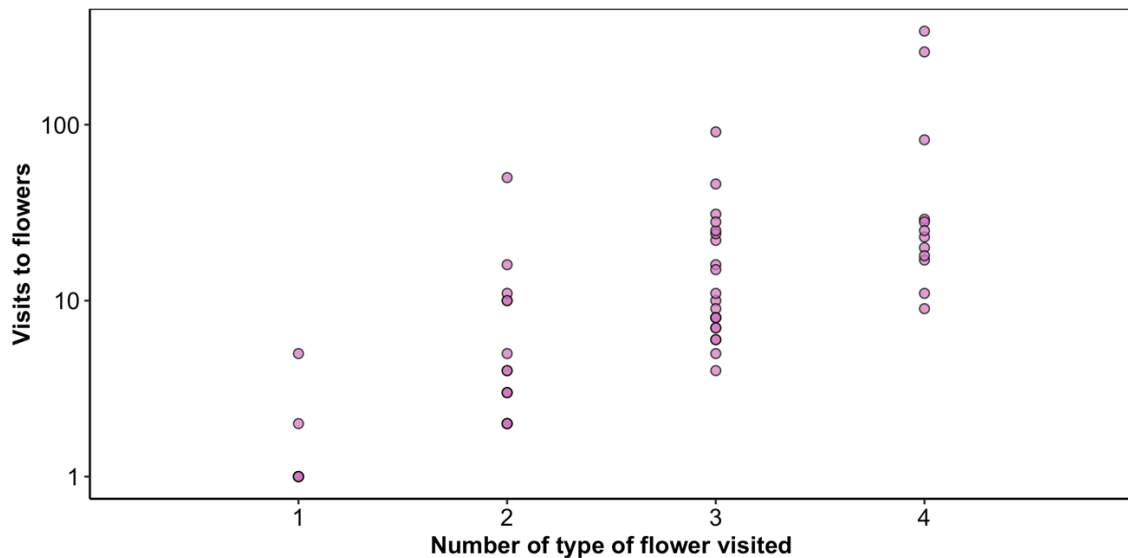


Figure 16: Overall distributions of visits to flowers by individual bees. Total visits (in log scale) compared to numbers of visits to flowers of each type, for each subgroup of individuals.

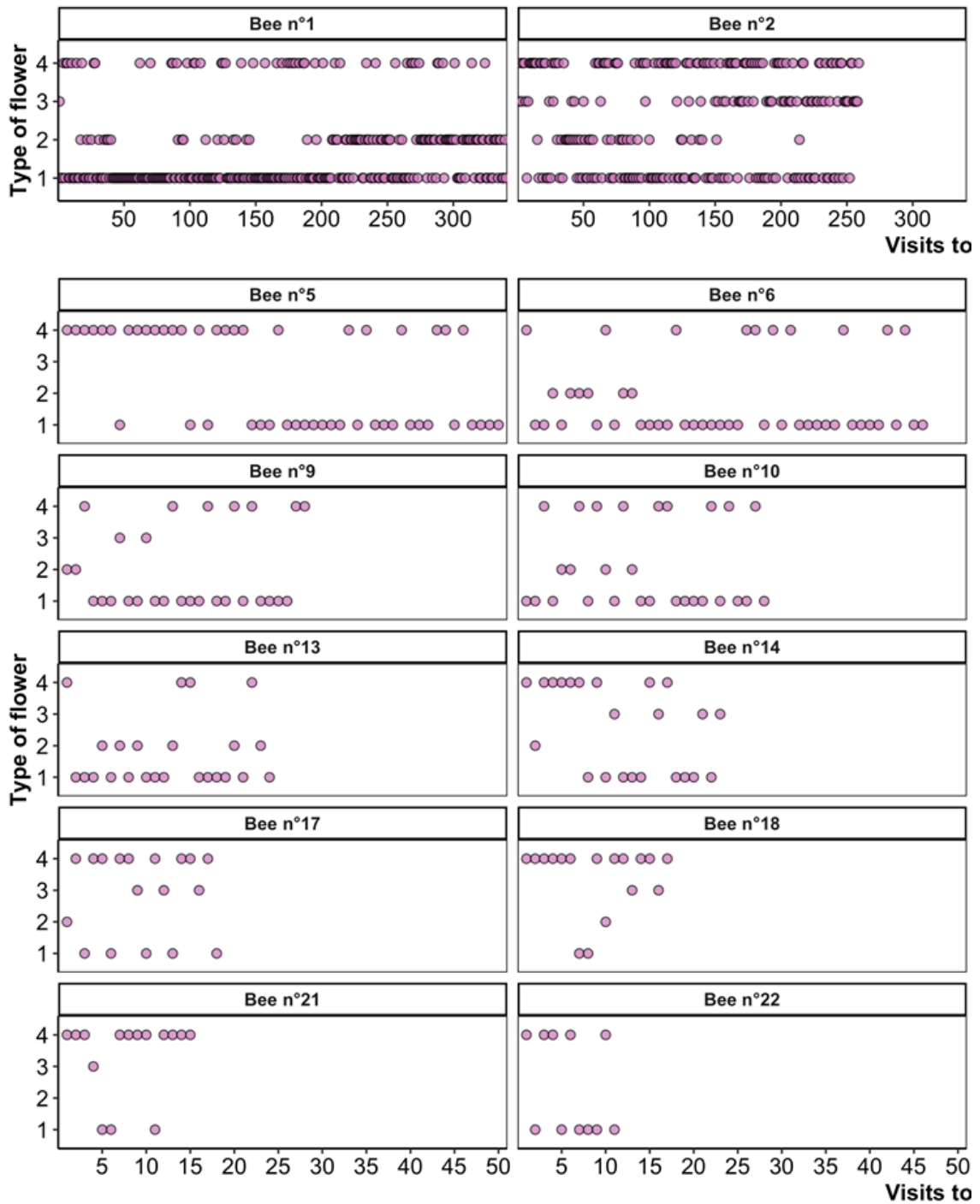
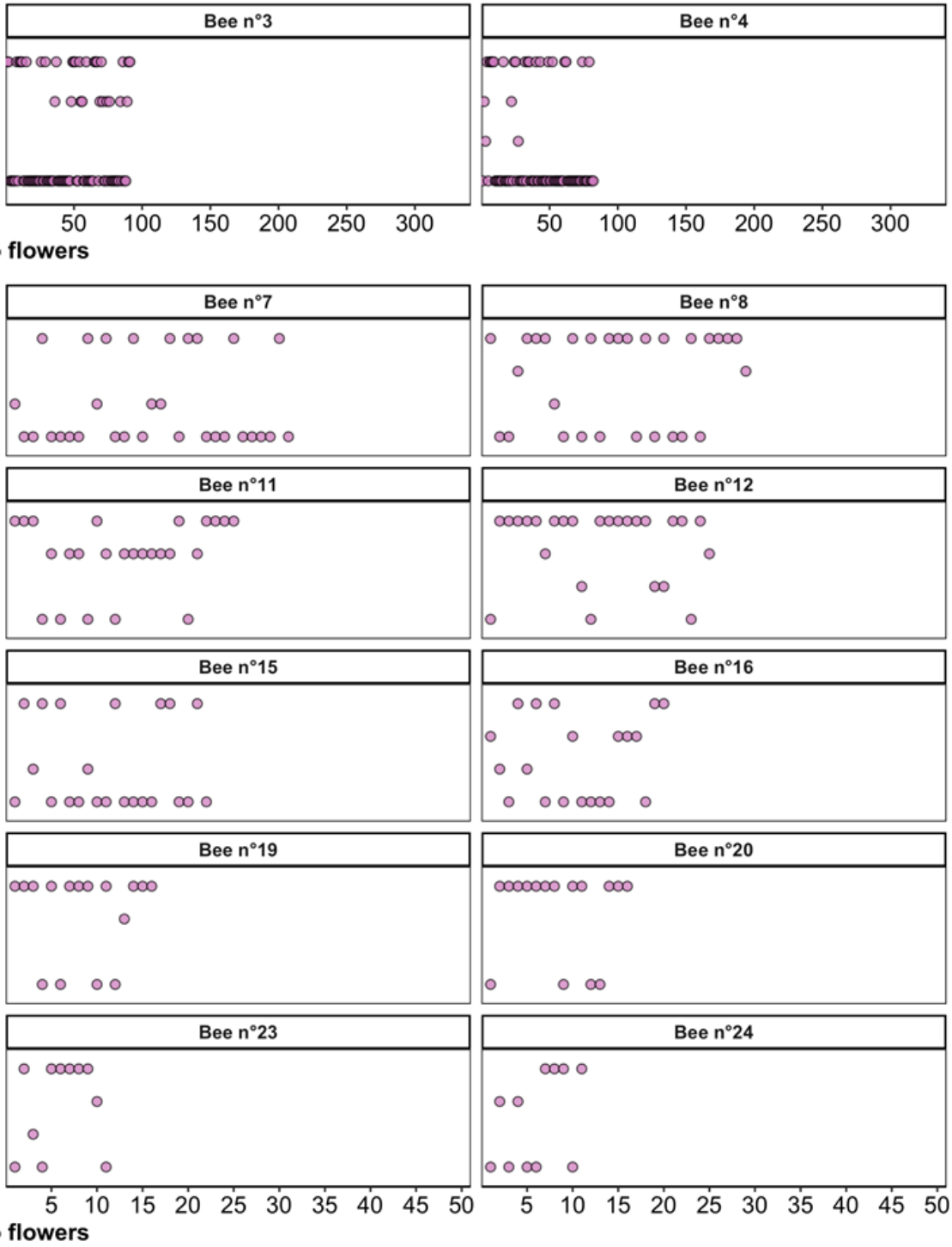
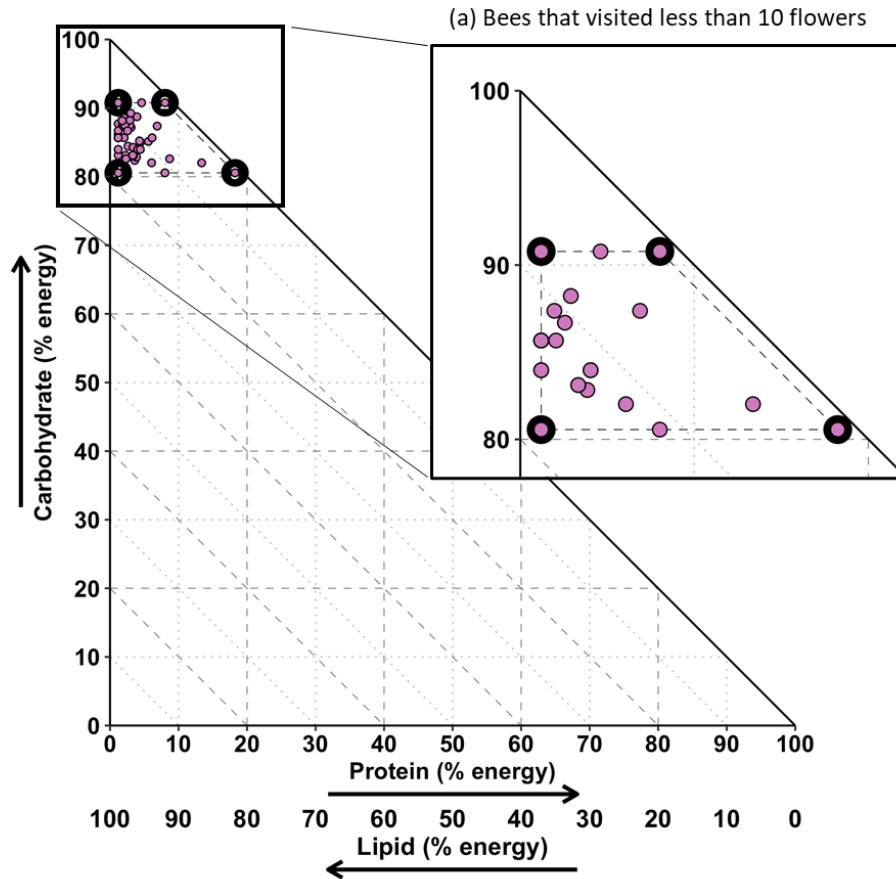


Figure 17: Individual visit patterns. Distribution of visits, among the four types of flowers and over time for each bee that made more than ten visits. The four upper bees are set apart due to high number of visit relative to other.





Bumblebees reach a similar nutrient ratio while foraging on artificial flowers

Bumblebees that made less than 10 visits ended on a diet of 83,97% [80.56;87.03] carbohydrates, 12.28% [6.74;14.21] lipids and 2.56% [1.2;6.31] proteins (Figure 18.a). Those that made 10 to 30 visits ended on a diet of 85.1% [82.86;87.53] carbohydrates, 11.35% [8.7;13.79] lipids and 2.9% [1.51;4.38] proteins (Figure 18.b). Those that made more than 30 visits ended on a diet of 87,81% [85,14;89,25] carbohydrates, 9.74% [8.5;13.13] lipids and 2.09% [1.2;5.59] proteins (Figure 18.c). These proportions were similar irrespective of the number of flower visits ($F_{(2,162)} = 1.73$, $p = 0.18$, $R^2 = 0.99$). Thus on average all bees reached a nutrient collection ratio of 2:88:10 (P:C:L) in our experiment.

(b) Bees that visited between 10 and 30 flowers (c) Bees that visited more than 30 flowers

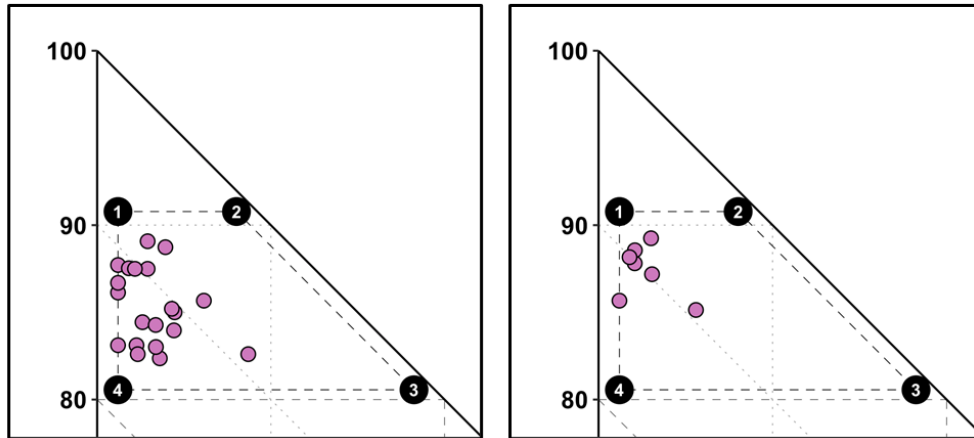


Figure 18: Nutrient collection on artificial flowers. Each dotted polygon represents the nutritional space corresponding to the four diets used in this experiment. Coloured dots are the ended nutrient collection for each forager, for each subgroup: **(a)** bees that visited less than 10 flowers; **(b)** bees that visited between 10 and 30 flowers; **(c)** bees that visited more than 30 flowers.

Conclusion

We observed that bees in a social foraging on artificial flowers in outside condition behaved as nutrient generalists. As the number of flower visits increased, they converged onto a common nutritional balance.

Chapter Discussion

Previous experiments showed individual bees can regulate their nutrient collection from artificial diets (Stabler et al., 2015; Vaudo et al., 2016b). Here we were interested in identifying some factors that may drive these food choices in individual bees, such as the macronutrient content, the age or caste of individuals, or social influences. We found that bumblebees are generally more responsive to carbs in food than lipids and proteins. All bees in the colony seem to have a similar nutrient collection target irrespective of age and body size, that they can achieve individually by being flower generalists.

More bumblebees responded to carbohydrates over lipids and proteins

In the first study, using PER assays, we observed a greater proportion of bumblebees responding to carbohydrates than to lipids, and even more than to proteins. This result confirms our main hypothesis, consistently with the preference for a high-sugar diet previously observed in small groups of bumblebees in laboratory assays (Stabler et al., 2015). The higher response rate to lipids than to proteins was more surprising, since adult workers usually require mainly carbohydrates with low amounts of proteins for somatic maintenance, in several social insect species (honeybees: Pirk et al., 2010; Altaye et al., 2010; Paoli et al., 2014a; ants: Dussutour and Simpson, 2009). Indeed, a previous study based on a choice between multiple diets varying in both lipids and proteins found that *B. terrestris* and *B. impatiens* workers converged on a diet containing respectively 14 and 12 times more proteins than lipids (Vaudo et al., 2016b). The observed high mortality rate constrained on high-lipid diets could explain the preference for low-lipid diets. However, as the authors mentioned, it is unclear if survival decreased because of insufficient protein intake or of a toxic excess of lipids. Nevertheless, it is difficult to compare their results to ours as both studies used diets made up of different components, knowing that nutrient sources can influence the nutritional intake of bumblebee workers (Stabler et al., 2015).

Bees responded to lower concentrations of lipids compared to carbohydrates

Among bumblebees that responded, the majority of those tested on lipids first responded to the lowest concentration (0.1%), while those tested on carbohydrates started responding mostly to 1, 3 or 10%. This likely reflects a high sensitivity to lipids, consistently with another study demonstrating that bumblebees discriminated better pure pollen from pollen added with fatty acids, than to pollen added with amino-acids (Ruedenauer et al., 2016). This high sensitivity could facilitate avoiding an over-consumption of lipids that might be toxic as seen before (Vaudo et al., 2016b; Ruedenauer et al., 2020).

However, here the low response threshold to lipids was observed by monitoring PER, an appetitive response, indicating that not only did they perceive fatty acids in the diet, but was also that they were motivated to consume them.

Indeed, lipids still remain crucial for pheromonal communication (Ayasse and Jarau, 2014) and wax production (Tulloch, 1970). Also, specific lipids are beneficial to both individuals and colonies. For example, memory and survival performances increase with the addition of oleic acid in diet (Muth et al., 2018), while sterol levels in pollen have been correlated to colony health and fitness (Vanderplanck et al., 2014; Moerman et al., 2017), both present in our lipid mix. Thus, sensing lipids in pollen appears to be primordial for bumblebees, but their consumption needs to be limited (Vaudo et al., 2020). This may thus explain the low response threshold observed here. In contrast, workers are selective for sugars, and therefore their higher threshold for carbohydrates may reflect their preference for high concentrations (Martinez and Farina, 2008). In addition, the diet history of each individual could greatly influence its responses; e.g., if it previously lacked one macronutrient, it might show an increased response to it (Ruedenauer et al., 2016). Our micro-colonies were identically provided with food, but we could not control if all individuals in a micro-colony ate equally before experiments.

Lipids and proteins did not influence carbohydrates responses

As mentioned above, lipids and proteins are necessary in small amount in adult workers, so we could have expected a positive influence on the carbohydrate's response.

However, bumblebees' responses to carbohydrates remains similar over the increasing addition of either lipids or proteins. As excess of lipids and proteins could be deterrent for insects (Dussutour and Simpson, 2012; Vaudo et al., 2016b; Ruedenauer et al., 2020), the opposite expectation that adding them to a carbohydrate basis would decrease the intake as their concentration increases is possible. But again, this did not affect the response of bumblebees to carbohydrates in our experiment. A possible explanation is that, because they were starved before the experiment, their need for energy intake might overcome the potential toxicity of additional nutrients, all the more when in low quantities.

Nutritional regulation was neither size- nor age-dependent, but younger bees tended on a richer protein diet

Results above were obtained on workers of unknown age and observed variability could be an age-dependent effect, which is well known to influence nutritional preferences in honeybees (Paoli et al., 2014a). When bumblebees of different ages could choose between diets varying in their macronutrient contents, they collected similar amounts of food irrespective of their age.

Still, younger bees tended to converge on a diet slightly richer in proteins, approaching the queen's diet preference tested in the same experiment. As they undergo maturation after emergence (Skandalis et al., 2011; Jones et al., 2013), they might have a greater need for proteins in the first days of adult life. On the other hand, older bees showed a slightly overall food collection with more carbohydrates but similar proteins intakes. Activity increase would increase sugar demands; however, it is not known if activity level is correlated with age. Finally, all bees in this experiment tended to converge to a specific lipid ratio, that supports the importance of lipid regulation discussed above (Vaudo et al., 2016a,b; Ruedenauer et al., 2020)

Rather than age, size is a major explanation to the task repartition in bumblebees (Goulson et al., 2002; Spaethe and Weidenmüller, 2002; Gardner et al., 2007; Jandt and Dornhaus, 2009; Tobback et al., 2011). Yet, we observed no influence of body size in neither of our experiments (note that body size was measured in the foraging

experiment, but its potential effect was not analysed due to the small dataset). Nonetheless, size variation in bumblebees is important, and smaller bees tend to stay in-hive for maintenance while larger bees tend to be the foragers (Goulson et al., 2002; Spaethe and Weidenmüller, 2002). This task repartition by size let us hypothesize there will be an effect on nutrient perception and preference, but we could not provide it.

Foragers are nutritional generalist, visiting multiple flowers to balance their nutrient collection

After observing that in laboratory bumblebees were able to non-randomly select between food to achieve a specific nutritional balance, we wanted to confirm this result in a more ecological setup while looking at differences among foragers. In our conditions, we observed that free-flying foragers did not focus on a single type of flower, while it is well known that pollinators, including bumblebees, often exclusively visit a certain flower type and stick to it, a behaviour known as flower constancy (Free, 1970; Heinrich et al., 1977). Yet, flower constancy is not a strategy used in all circumstances. Indeed, a previous study observed the foraging pattern of bumblebees in laboratory conditions, where they could fly among 60 to 80 flowers varying in colour, shape, size and odour.

When flowers differed in multiple traits at the same time (*e.g.*, colour and shape or colour and odour), bumblebees restricted their visits to one type of flowers and thus performed flower constancy. However, they visited multiple types of flowers when they varied only by colour (Gegear and Laverty, 2005). The use of flower constancy was thus hypothesized as a consequence of the limited capacity to remember multiple traits at the same time, allowing to focus sequentially on different flower types (each type for some time) (Chittka et al., 1999; Goulson, 2000). Consistently, in our conditions most bumblebees were not constant in their flower visits, as artificial flowers containing different diets could be discriminated only by colour. Rather, they were able to combine several diets on multiple flowers to achieve a specific nutritional intake when foraging. This nutritional behaviour, essentially studied in laboratory conditions (Stabler et al., 2015; Vaudo et al., 2016b; Kraus et al., 2019a), reveals that bumblebees were able to

discriminate and remember (based on colour cues) the different diets. We found no significant effect of the number of visits on the proportion of carbohydrates, lipids and proteins collected, and foragers tended to converge on a common nutritional balance while continuing to balance between flowers all over the experiment. Still, it remains unclear if bumblebees could specialize on specific nutrient intake, since here foragers that collected more proteins were among those visiting few flowers. We could not conclude whether this was a specific choice (in which case they would have kept to it over subsequent visits if they had made more) or if, with more visits, they would focus on other flowers and thus obtained a similar nutritional diet as other foragers. The possibility remains that bumblebees that collected mostly proteins did so temporarily to respond to a temporary need from the colony, and that they did not need to do many visits because proteins are usually required in lower amounts than carbohydrates (Stabler et al., 2015), thus reflecting in less visits dedicated to collecting pollen than nectar (Russell et al., 2017a).

Concluding remarks and perspectives

These experiments provided interesting preliminary results but remain incomplete and deserve to be pursued in future studies. Tracking more foragers on subsequent days would give us the opportunity to conclude on a possible specialisation in nutrient collection that could be explained via a nutritional preference in foragers. It is possible that such specialisation is observed on a daily basis, but changes over the week as previously observed on foraging RFID-tagged bumblebees, which focused on nectar or pollen over a day (Russell et al., 2017a). Furthermore, bumblebees could be then caught and submitted to a nutrient sensitivity test as in our first study, to see if there is a correlation between their score and their visit patterns.

Besides, the same experiment could be performed on foragers returning to their hive. We hypothesize that putative pollen specialists (caught with pollen loads on their legs) would have a lower probability to respond to proteins and lipids, contrary to putative nectar specialists. This assumption was observed in honeybees, where pollen foragers responded to lower concentrations of sucrose in honeybees (Page et al., 1998), probably

if nutritional preference is driven by appetitive motivation, nectar foragers lose their sensitivity to sucrose while pollen foragers not. Testing bumblebees before and then releasing them to track their foraging pattern could allow us to confront their nutritional preferences and their flower specialisation.

Ultimately, perceiving how bumblebees sense the different nutrients in food and how they regulate their collection will give us an insight on their foraging pattern and collective organisation for nutrient collection. Selecting the appropriate amount and balance of each nutrient is critical for foragers to respond to varying contexts that occur, either inside their colony with larval production and competition between workers, or outside with environmental change through season and climate change.

[Zazu : Didn't your mother ever tell you not to play with your food?]

-- *The Lion King (1994)*



CHAPTER 2

BUMBLEBEES ADJUST PROTEIN AND
LIPID COLLECTION RULES TO THE
PRESENCE OF BROOD

CHAPTER 2: Bumblebees adjust protein and lipid collection rules to the presence of brood

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Summary:

- Here we used a 3D nutritional geometry design to examine how bumblebee workers regulate their collection of 3 major macronutrients in the presence and absence of brood.
- Colonies given a choice between nutritionally complementary diets selected foods to reach a target ratio of 71% proteins, 6% carbohydrates, and 23% lipids, irrespective of the presence of brood.
- When confined to a single nutritionally imbalanced solid diet, colonies without brood regulated lipid collection and over-collected protein relative to this target ratio, whereas colonies with brood regulated both lipid and protein collection.
- This brood effect on the regulation of nutrient collection by workers highlights the influence of the social context on workers' behaviours.

Key words: artificial diets; *Bombus terrestris*; bumble bees; nutritional geometry; rule of compromise

Introduction

Animals have evolved behavioural strategies to acquire nutrients in amounts and balances that maximize fitness traits, such as development (Jang and Lee, 2018), metabolic health (Solon-Biet et al., 2015), reproduction (Maklakov et al., 2008), and lifespan (Piper et al., 2014). These strategies are best understood using nutritional geometry (Raubenheimer and Simpson, 1993; Simpson and Raubenheimer, 1993; 2012), a modelling approach that describes how animals can reach and maintain an optimal nutritional state (the intake target) by adjusting their food consumption to their needs. Depending on foods available in the environment, animals can either select a nutritionally balanced food that led them directly to their intake target, mix their intake of several individually imbalanced but complementary foods, or consume imbalanced foods according to a rule of compromise between over-ingesting some nutrients and under-ingesting others (for recent reviews see Simpson et al., 2015; Raubenheimer and Simpson, 2018).

In animals whose food selection depends on social interactions, these nutritional decisions are complicated (Lihoreau et al., 2014; 2015). The challenge of group nutrition is exemplified in social insects, where foragers must balance their collection of foods to reach a colony level intake target that addresses the divergent needs of all colony members, including the non-foraging workers, the brood (larvae), and the reproductive males and females (Lihoreau et al., 2018). In these superorganisms, workers typically require carbohydrates for energy (honeybees: Paoli et al., 2014a; bumblebees: Stabler et al., 2015; ants: Wills et al., 2015) whereas queens and larvae primarily need proteins and lipids for egg-laying and development (honeybees: Pirk et al., 2010; bumblebees: Stabler et al., 2015; ants: Dussutour and Simpson, 2009). Nutritional geometry studies have shown that social insect foragers mix their collection of nutritionally complementary foods to reach a colony target for protein and carbohydrates (ants: Dussutour and Simpson, 2009; Cook et al., 2010; Shik et al., 2016; honeybees: Hendriksma and Shafir, 2016). In ants, this target ratio varies with feeding habits and colony composition, with foragers collecting more protein in the presence of brood

(Dussutour and Simpson, 2009) or symbiotic fungi (Shik et al., 2016). The ability of foragers to adjust nutrient collection to the collective needs is vital for the colony. At the physiological level, an excess of protein (or amino-acids) reduces lifespan (honeybees: Pirk et al., 2010; Paoli et al., 2014b4; ants: Dussutour and Simpson, 2012; Arganda et al., 2017), whereas a high carbohydrate intake has detrimental effects on larval growth and survival (Helm et al., 2017). At the behavioural level, deficits in specific dietary lipids (e.g., omega-3) alter learning performances required for foraging (honey bees: Arien et al., 2015; 2018), a harmful effect that can potentially lead to colony collapse (Klein et al., 2017).

For bees, nutrient balancing is particularly challenging as it involves exploiting highly diverse, ephemeral and spatially distributed floral resources, in the form of liquid nectars and solid pollens. Nectar is a major source of water and carbohydrates, whereas pollen mainly contains protein, lipids, free amino-acids, and other micronutrients (Wright et al., 2018). Experiments using artificial diets have shown that social bees can individually and collectively balance their acquisition of 2 nutrients simultaneously. Individual workers, or small groups of workers, balance their intake of carbohydrates and protein (or free amino-acids) in nectar (honeybees: Altaye et al., 2010; Pirk et al., 2010; Paoli et al., 2014a; bumblebees: Stabler et al., 2015), as well as protein and lipids in pollen (bumblebees: Vaudo et al., 2016a,b). The full-size colonies can also balance their intake of essential amino acids in pollens (honeybees: Hendriksma and Shafir, 2016).

Although this is an important first step, bee colony nutrition involves collecting both nectars and pollens, and therefore regulating the acquisition of more than 2 nutrient sources simultaneously. Recent studies using 3D nutritional geometry designs show how taking into account more than 2 nutrients, and their natural associations in foods, can help bring new insights into the fundamental nutritional biology of species that would otherwise be overlooked; for instance, to explain why animals prioritize the regulation of some groups of nutrients over others (cats: Hewson-Hughes et al., 2011; mice: Solon-Biet et al., 2014) or do not appear to regulate nutrient intake at all (termites: Poissonnier et al., 2018).

Here, we used a 3D nutritional geometry design to examine how small colonies of bumblebees (*B. terrestris*) regulate their collection of protein, carbohydrates, and lipids, and how any impediment to do so affects their physiology. First, we investigated the ability of bumblebee colonies to freely regulate their nutrient collection from artificial liquid and solid diets, in the presence or absence of brood. Second, we explored the influence of brood on the nutrient collection rule of compromise used by bumblebees when confined to a single imbalanced diet. Third, we examined the effects of nutrient intake on adult emergence rate, body lipid composition and lifespan. We used artificial diets with extreme ranges in nutrient contents reported in natural foods to investigate to what extent bees can balance their nutrient collection and use rules of compromise in highly challenging conditions.

Material and Methods

Bumblebees

Experiments were conducted between January and April 2016. Thirty-one commercial colonies of *B. terrestris* (Biobest, Orange, France) were used. Each colony contained about 200 workers, brood, and 1 queen. Bumblebees were maintained and tested in the laboratory at 25°C and 30–40% relative humidity, under a 12:12 h light:dark photcycle. From these mother colonies, 136 microcolonies of 20 workers were set up (i.e., 4–5 microcolonies per mother colony). All workers of a given microcolony originated from the same mother colony. Each microcolony was placed in a plastic box divided in 2 compartments of the same size (Figure 19). The nest compartment was dark and contained 30 nest cells (either 30 empty cells or 20 empty cells plus 10 cells containing larvae, see details in Table 5). For each microcolony, brood and empty cells were taken from the same mother colony as the workers. Brood was in pupal stage (between 3- and 5-weeks old). Ad libitum water was provided in a gravity feeder (vertical 15 mL tube with 2 holes at the bottom from which the bumblebees could insert their proboscis and drink)

in the nest compartment to avoid interfering with nutrient collection in the foraging compartment (i.e., limited access to nectar and pollen diets due to overcrowded conditions). The foraging compartment was clear and connected to 3 small feeding boxes (Figure 19), each containing either artificial nectar or pollen (see details below). Nectar was provided by 4 gravity feeders (15 mL) per feeding box. Pollen (1.5 g) was provided on 10 vertical chenille stems (2.5 cm) per feeding box. We used chenille stems to mimic flower stamen and facilitate collection by bees, as recommended by Russell and Papaj (2016).

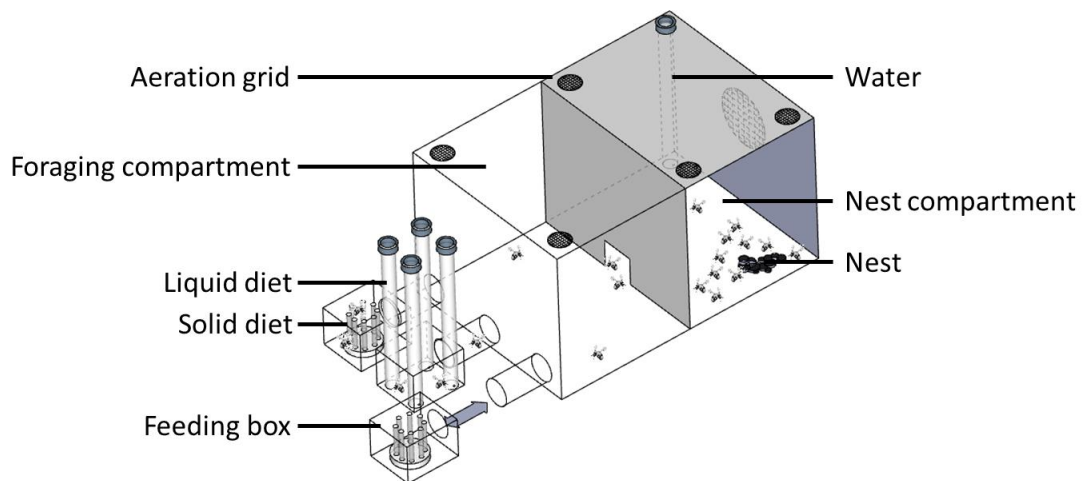


Figure 19: Schematic view of an experimental box used for brood influence experiment. Microcolonies of 20 bumblebee workers were kept with nest materials (either 30 empty cells or 20 empty cells with 10 cells containing larvae). Bumblebees could collect ad libitum water from a gravity feeder in the nest compartment, synthetic liquid diet (from gravity feeders) and solid diet (from chenille stems) in removable feeding boxes, for 13 days. Dimensions of the experimental box: 17.2cm (length), 11.6 cm (width), and 9.4cm (height). Dimensions of a feeding box: 5.7cm (length), 5.7cm (width), and 2.6cm (height). See pictures in Supplementary Figure S1.

Table 5: Summary of the choice and the no-choice experimental designs.

Experiment	Duration (days)	Solid diet	Liquid diet (%)	Mean number of empty cells at the beginning (95% CI)	Mean number of larvae at the beginning (95%CI)	Sample size behavioural assays (micro-colonies)	Sample size lipid body content (bees)
Choice	13	PI and pL	N10	27.9 (24.9-30.9)	0	10	13
			N60	28.1 (25.9-30.3)	0	10	12
			N10	19.1 (16.9-21.3)	10.3 (9.1-11.5)	10	14
			N60	17.4 (15.0-19.9)	10.5 (9.3-11.7)	10	15
No-choice	13	pL	N10	28.3 (26.4-30.1)	0	8	10
			N60	27.0 (25.0-29.0)	0	8	15
			N10	17.9 (14.9-20.9)	10.0 (8.6-11.4)	8	13
			N60	17.6 (15.3-20.0)	10.0 (9.0-11.0)	8	6
		PI	N10	27.0 (25.7-28.3)	0	8	5
			N60	26.8 (25.1-28.5)	0	8	12
			N10	18.6 (15.6-21.5)	9.7 (8.8-10.6)	8	6
			N60	17.3 (15.1-19.5)	10.1 (8.8-11.5)	8	11
		PL	N10	28.9 (26.6-31.2)	0	8	12
			N60	27.5 (25.3-29.7)	0	8	6
			N10	19.0 (15.8-22.2)	10.8 (9.1-12.4)	8	8
			N60	19.6 (17.2-22.1)	10.6 (8.8-12.4)	8	18

Artificial diets

The microcolonies were fed artificial nectars and pollens (hereafter referred as “liquid diet” and “solid diet”). Liquid diets consisted in sucrose solutions of 2 concentrations (w/w): 10% (N10%) and 60% (N60%). Solid diets were dry powders composed of lipids, protein, cellulose, and micronutrients. Three types of solid diets varying in their ratio of protein to lipids were used: low-protein, high-lipid diet (pL); high-protein, low-lipid diet (PI); and high-protein, high-lipid diet (PL) (see details in Table 6). Nutrient selection was made based on commonly used insect diets (Cohen 2015). The protein content of all the solid diets consisted of a mixture of casein (80%) and whey protein (20%) (Nutrimuscles, Longwy, France). The fat content consisted of a mixture of linseed oil (80%) (Sigma-Aldrich, Darmstadt, Germany) and cholesterol (20%) (Sigma-Aldrich, Darmstadt, Germany). Linseed oil was chosen for its high concentration in omega-3 (56%) which is important for brain functions (Arien et al., 2015). Cholesterol was chosen as the main source of sterols because it is essential to bees and many other sterols found in natural pollens are used for its synthesis (Herbert et al., 1980; Behmer and Nes, 2003). Micronutrient contents consisted of a mixture of vitamins (25%) (Vanderzant vitamins mix, Sigma-Aldrich, Darmstadt, Germany), choline chloride (25%) (Sigma-Aldrich, Darmstadt, Germany), inositol (25%) (BVBA Acros Organics, Geel, Belgium), and ascorbic acid (25%) (Fisher Scientific, Illkirch-Graffenstaden, France). Cellulose, a non-digestible

carbohydrate for bees (Roulston and Cane, 2000), was used as binding agent (Sigma-Aldrich, Darmstadt, Germany). To produce the diets, we first added cholesterol into linseed oil, then protein and micronutrients, and finally the cellulose while mixing. Cellulose addition into lipids produces a homogenous powder substance.

We designed artificial diets with maximum variation in nutrient ratios, within ranges reported in natural nectars and pollens (nectars: Nicolson and Thornburg, 2007; pollens: Rothnie et al., 1987; Roulston and Cane, 2000; Somerville and Nicol, 2006; Nicolson, 2011; Vaudo et al., 2016a), to investigate the ability of bees to balance their nutrient collection and adjust rules of compromise in highly challenging nutritional conditions. We did not use a low-protein low-lipid diet (pl) because such diet would not yield enough nutrients for bees to reach any nutrient collection target or express rules of compromise. Preliminary observations showed that bumblebees could collect all our diets and store them in empty brood cells.

Table 6: Recipes of the three artificial solid diets used in the brood influence experiment. Proportion (%) of proteins, lipids, digestible carbohydrates, cellulose and micronutrients in the 3 artificial solid diets (pL, Pl, and PL).

Ingredients (/100g)	pL	Pl	PL
Casein	0.8	40	40
Whey protein	0.2	10	10
Linseed oil	16	0.8	16
Cholesterol	4	0.2	4
Vitamins mix	0.5	0.5	0.5
Choline chloride	0.5	0.5	0.5
Inositol	0.5	0.5	0.5
Ascorbic acid	0.5	0.5	0.5
Cellulose	77	47	28
Total Proteins	0.835	41.75	41.75
Total Carbohydrates	0.0326	1.63	1.63
Total Lipids	19.997	1.74	20.69
Total Micronutrition	2.041	3.876	3.88

Behavioural assays

Two experiments were run. In the “choice” experiment, microcolonies were given a choice between 1 type of liquid diets (N10% or N60%) and 2 types of solid diets (PI and pL). We used PI and pL to provide bees the opportunity to balance their diet in the broadest possible nutrient space. In the “no-choice” experiment, microcolonies were fed 1 type of liquid diet (N10% or N60%) and 1 type of solid diet (pL, PI, or PL). Nutrient collection by microcolonies with brood and without brood was compared. Eight to 10 microcolonies were tested per condition (see summary in Table 5). Each experiment was conducted during 13 consecutive days. Solid and liquid diets were renewed every 2–3 days by replacing the feeding boxes by new boxes containing fresh diets (Figure 19). This manipulation enabled the collection of liquid and solid diets by the bumblebees (milligrams/bumblebee) to be recorded. Collection of liquid diet was quantified by measuring the volume of diet remaining in the gravity feeders (centimetres).

Collection of solid diet was quantified by measuring the weight of diet remaining on the chenille stems (milligrams) (entire feeding boxes were weighted, including the petri dish and chenille stems). The solid diet was dried (65°C for 48 h) before being weighed with a precision scale (± 0.001 g; Mettler Toledo, Greifensee, Suisse). This protocol only permitted to measure food collection by bumblebees, not consumption per se. In parallel to the experiments, liquid and solid diets were placed in empty setups (feeding boxes connected to empty nest boxes) to measure volume and weight changes due to evaporation and correct the experimental measures. Sixteen of these controls were used for each type of liquid diet (N10% and N60%) and solid diet (pL, PI, and PL). Every day, dead bumblebees were counted, removed from the boxes, and frozen at -20°C for lipid analyses (see below). For each microcolony, the number of new-born adults (i.e., emerged from the brood cells), N_b , was calculated as follows:

$$N_b = N_l - (N_d + N_{lp})$$

where N_l is the number of live individuals, N_d is the number of dead individuals, and N_{lp} is the number of live individuals during the previous recording.

Lipid analyses

Body lipid compositions were quantified using chloroform extraction (Cook et al., 2010; Dussutour et al., 2016; Arganda et al., 2017). Dead bumblebees were dried (65°C for 48 h) and weighed (dry mass). To extract the whole-body fat content, bumblebees were soaked in chloroform for 3 days (chloroform was changed every 24 h), dried and weighed again to obtain the body mass without lipids (lean mass). Only the bumblebees that died between Days 5 and 13 after the beginning of the experiments were used to make sure that the main variations in body composition measured were caused by variations in the nutrient contents of the artificial diets consumed. A total of 176 bumblebees (5–18 randomly chosen individuals per test condition) were analysed (Table 5).

Statistical analyses

All statistical analyses were performed in R 3.5.1 (R Core Team 2018). From the raw liquid and solid diet collection data (weight of diet collected), average carbohydrate (C), protein (P), and lipid (L) collection by bumblebees per day were calculated:

$$N = \frac{\sum Di \times Pi}{T \times Nl}$$

where N is the total amount of the focal nutrient collected (C, P, or L); Di is the amount of diet i collected (weight in milligrams); Pi is the proportion of N in Di; T is the time since the beginning of the experiment (in days), Nl is the number of live bumblebees in the colony at T.

For the choice experiment, we tested how the average amount of P, C, and L collected by bumblebees per day were affected together by the liquid diet type (N10%, N60%) and brood (presence, absence), using a multivariate analysis of variance (mixed-effects MANOVA, function `manova` in package “stats” (R Core Team 2018)). The effects of food type (liquid diet, solid diet pL, and solid diet Pl), liquid diet type, and brood on the average amount of food collected by bumblebees per day were tested with an analysis of variance (mixed-effects ANOVA, function `anova` in package “stats”).

For the no-choice experiment, the effects of solid diet type (pL, Pl, PL), liquid diet type, and brood on the average amount of food collected, the average amount of lipid collected, and the average amount of protein collected by bumblebees per day were tested with a mixed-effects ANOVA (function `anova` in package “stats”).

For both choice and no-choice experiments, the relationship between the amount of nutrients collected per day and the number of empty brood cells in the nest were tested using an analysis of covariance (mixed-effects ANCOVA, function `anova` in package “stats”).

The effects of solid diet type, liquid diet type, and brood on adult emergence rate and survival were assessed using a Cox proportional hazards regression model (function `coxph` in package “survival”, Therneau and Lumley, 2015).

The effects of solid diet type, liquid diet type, and brood on body lipid composition across conditions were tested with a mixed-effects ANOVA (function `anova` in package “stats”).

All statistical models accounted for any possible mother-colony effect by adding mother-colony identity as random effect. Multiple comparisons were performed with post hoc Tukey Honestly Significant Difference (HSD) tests (function `glht` in package “multcomp”, Hothorn et al. 2016).

Results

Choice experiment

To quantify the nutrient collection target of bumblebees (i.e., amounts and balances of nutrients collected by workers and maximizing colony fitness) in the presence or absence of brood, microcolonies were given a choice between the solid Pl and the solid (pL), with either low-carbohydrate liquid diet (N10%) or high carbohydrate liquid diet (N60%) (Table 5).

On average, bumblebees collected more Pl (3.92–5.76 95% confidence interval [CI], N = 40 colonies), than pL (2.22–3.46 95% CI, N = 40 colonies) and liquid diet (0.13–0.21 95% CI, N = 40 colonies) (Figure 20.a; ANOVA, food type: $F_{2,85} = 97.72$, $p < 0.001$). This pattern was similar in all test conditions, irrespective of the type of liquid diet used and of the presence of brood in the nest (ANOVA, liquid diet: $F_{1,85} = 0.20$, $p = 0.66$; brood: $F_{1,85} = 0.01$, $p = 0.99$; all interactions: $p > 0.05$). There was no significant relationship between the daily amount of food collected by bumblebees and the number of empty brood cells in the microcolonies (ANCOVA: empty cells: $F_{1,225} = 1.17$, $p = 0.28$; empty cells x nectar: $F_{1,225} = 2.3$, $p = 0.13$). This means that differences in liquid and solid diet collection between choice conditions cannot be explained by a difference in space available to store food in the nest. When considering the nutritional composition of foods, microcolonies collected similar amounts of protein, carbohydrates and lipids irrespective of the type of liquid diet provided and of the presence of brood in the nest (MANOVA, liquid diet: $F_{3,11} = 1.55$, $p = 0.26$; brood: $F_{3,11} = 0.14$, $p = 0.93$; liquid diet x brood: $F_{3,11} = 2.95$, $p = 0.08$). In all choice conditions, bumblebees approached a nutrient collection ratio of 70.8% of protein (68.7–72.9 95% CI, N = 40 colonies), 6.2% of carbohydrate (5.4–7.0 95% CI, N = 40 colonies), and 23% of lipids (21.0–25.0 95% CI, N = 40 colonies) (Figure 20.b), as calculated by the percentage of each macronutrient out of the total amount of the 3 macronutrients (P, C, L). Therefore, in these choice conditions, bumblebees adjusted their collection of liquid and solid diets to a nutrient ratio that is independent of colony composition.

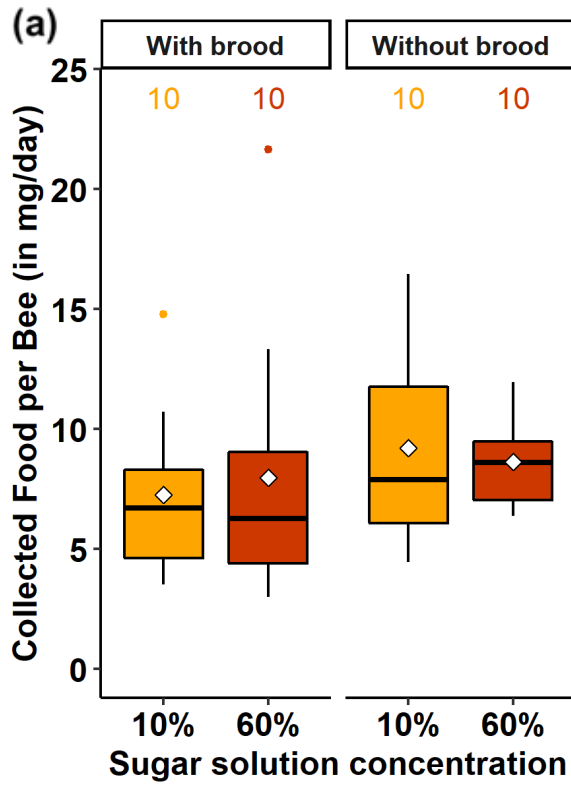
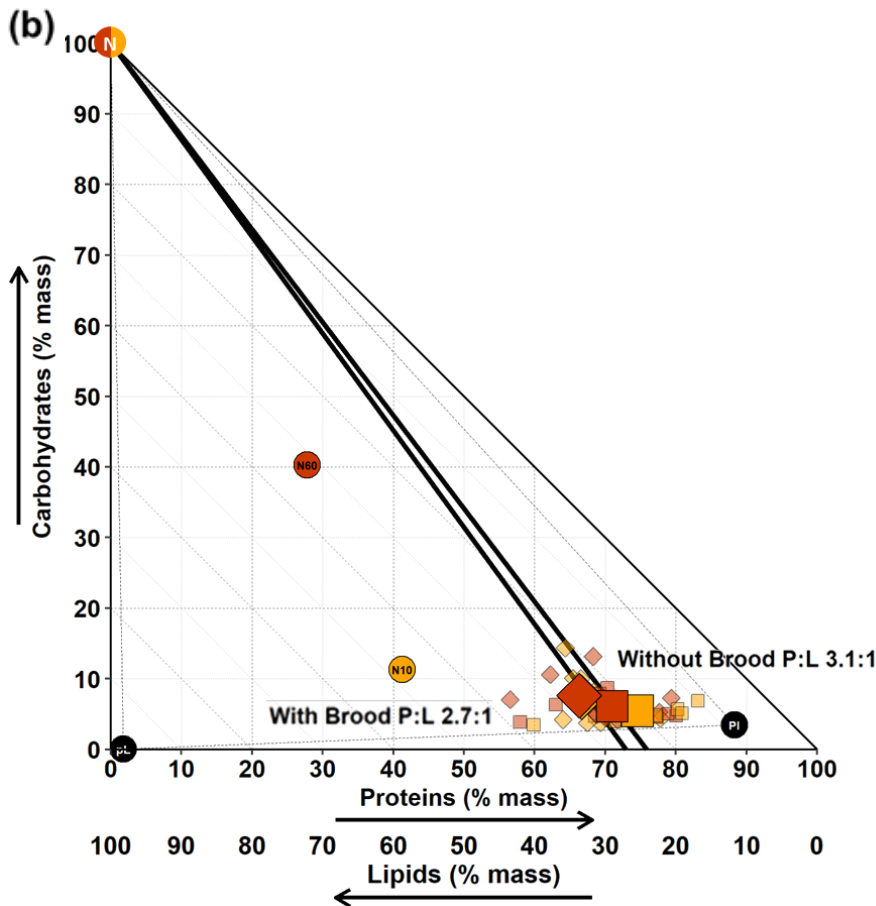


Figure 20: Food and nutrient collection in choice experiment. Microcolonies of 20 bumblebees with or without brood were observed foraging on 2 types of solid diets (PI, pL) and 1 type of liquid diet (10% or 60%) for 13 days. **(a)** Boxplot of the amount of liquid and solid diets collected each day per bee (milligrams). The central line is the median, the edges of the box are the 25th and 75th percentiles, the whiskers extend to the most extreme data points not considered outliers, outliers are represented by points, and diamonds represent means. **(b)** Nutritional geometry representation of the mean ratio of protein (P), carbohydrate (C), and lipid (L) collection by bumblebees. We calculated the average amount of each nutrient daily collected by bumblebees as the proportion of the focal nutrient divided by the proportion of that nutrient in liquid food and the 2 solid foods. Bars represent median P:L ratio. Eight to 10 microcolonies were used per test condition (see details about sample sizes in Table 5).



No-choice experiment

To explore the rule of compromise used by bumblebees to trade-off their collection of proteins, lipids, and carbohydrates from imbalanced foods, microcolonies were confined to 1 type of solid diet (PI, pL, or PL) and 1 type of liquid diet (low carbohydrate [N10%] or a high carbohydrate [N60%]) (Table 5). The total amount of food collected by bumblebees differed according to the type of solid diet and the presence of brood in the microcolony (Figure 21.a; ANOVA, solid diet: $F_{2,50} = 7.95$, $p < 0.01$; brood: $F_{1,50} = 9.88$, $p < 0.001$; solid diet x brood: $F_{2,50} = 5.12$, $p < 0.01$).

Liquid diet type had no effect (ANOVA, liquid diet: $F_{1,50} = 0.69$, $p = 0.41$; liquid diet x brood: $F_{1,50} = 0.74$, $p = 0.39$; solid diet x nectar: $F_{2,50} = 0.72$, $p = 0.49$; solid diet x liquid diet x brood: $F_{2,50} = 0.17$, $p = 0.84$) on food collection. Microcolonies without brood fed PI collected more food than microcolonies in all other conditions.

There was no significant relationship between the daily amount of nutrients collected and the number of empty brood cells between test conditions (ANCOVA: empty cells: $F_{1,566} = 0.34$, $p = 0.56$; empty cells x solid diet: $F_{2,566} = 0.4$, $p = 0.67$; empty cells x liquid diet: $F_{1,566} = 0.01$, $p = 0.94$; empty cells x solid diet x liquid diet: $F_{2,566} = 0.35$, $p = 0.7$), meaning that these differences could not be explained by a difference in space available to store the food in the nest. When considering the nutritional composition of foods, microcolonies showed different strategies in the absence and presence of brood. In the absence of brood, microcolonies fed high-lipid solid diet (pL or PL) collected similar amounts of lipids (Figure 21.b; ANOVA, solid diet: $F_{2,18} = 15.37$, $p < 0.001$; post hoc Tukey HSD, PL–pL: $p = 0.87$; PI–pL: $p < 0.001$; PL–PI: $p < 0.001$). However, this was not the case for microcolonies fed high-protein solid diets (PI or PL), as microcolonies fed PI collected more protein than those fed PL (Figure 21.b; ANOVA, pollen: $F_{2,18} = 14.62$, $p < 0.001$; post hoc Tukey HSD, PL–pL: $p < 0.05$; PI–pL: $p < 0.001$; PL–PI: $p < 0.01$). These results suggest that bumblebee workers prioritized lipid regulation over protein regulation, by over-collecting proteins to attain their lipid collection target. In the presence of brood, microcolonies fed high-lipid solid diets (pL or PL) collected a similar amount of lipids (Figure 21.c; ANOVA, pollen: $F_{2,18} = 15.55$, $p < 0.001$; post hoc Tukey HSD, PL–pL: $p = 0.67$;

Pl–pL: $p < 0.001$; PL–Pl: $p < 0.001$). This amount was comparable with that observed in microcolonies with no brood (Figure 21). Interestingly, microcolonies fed high-protein solid diets (Pl or PL) also collected similar amounts of proteins (Figure 21.c; ANOVA, solid diet: $F_{2,18} = 50.03$, $p < 0.001$; post hoc Tukey HSD, PL–pL: $p < 0.001$; Pl–pL: $p < 0.001$; PL–Pl: $p = 0.28$) suggesting that, in the presence of brood, bumblebees regulated both their collection of proteins and lipids to target values.

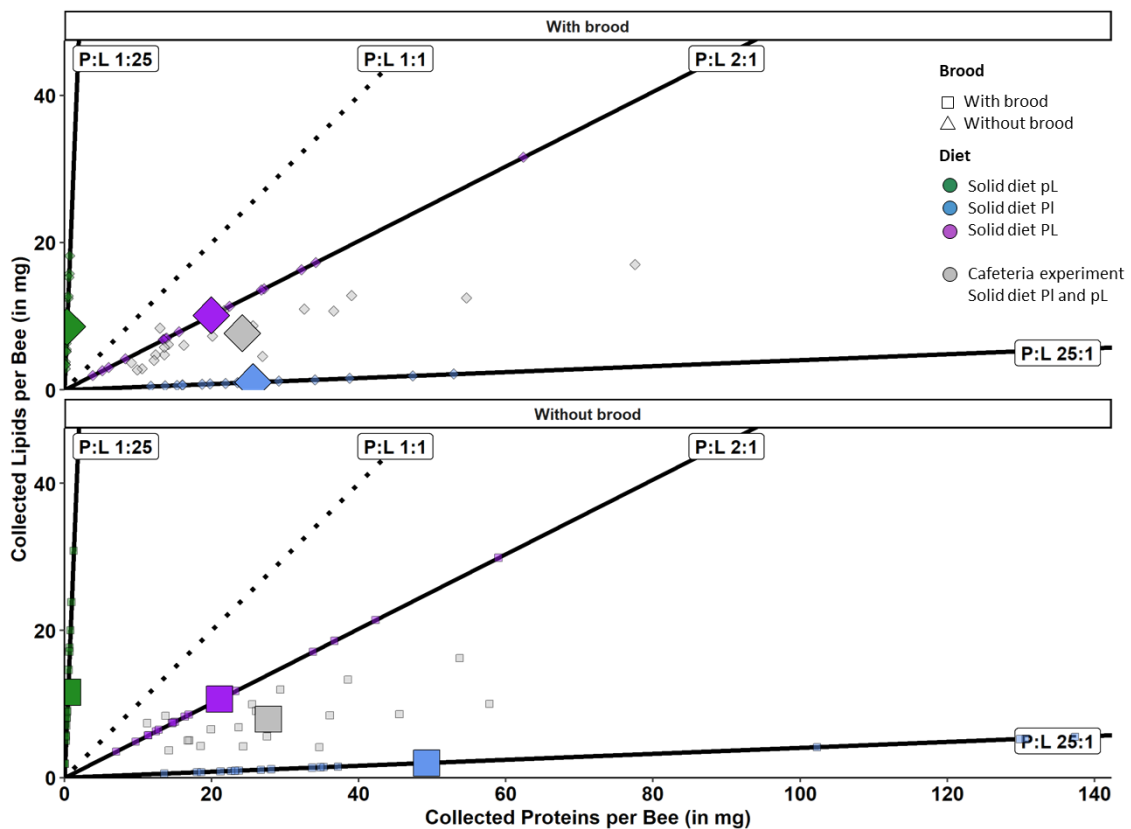


Figure 21: Food and nutrient collection in no-choice experiment. Microcolonies of 20 bumblebees with or without brood were fed 1 type of solid diet (Pl, pL, PL) and 1 type of liquid diet (10% or 60%) for 13 days. Mean collection of proteins (P) and lipids (L) per bumblebee day by day in microcolonies without brood or with brood. Colour symbols represent mean collection per day for each condition with or without brood in no-choice experiment. Gray symbols represent mean collection per day for each condition with or without brood in choice experiment. Lines represent the protein to lipid ratios of the diet colonies were fed. Eight to 10 microcolonies were used per test condition (see details about sample sizes in Table 5).

Survival

To assess the influence of diet on lifespan, we compared the survival of bumblebees in the different test conditions (Figure 22). In both the choice and the no-choice experiments, bumblebees survived longer when fed 60% liquid diet (N60%) than when fed 10% liquid diet (N10%) (Cox model, N60–N10: hazard ratio [HR] = 0.72; 95% CI 0.54–0.97; $p < 0.05$). The presence of brood had no effect on adult survival (Cox model, Brood–No brood: $H = 1.09$; 95% CI 0.78–1.50; $p = 0.62$). In the no-choice experiment, bumblebees fed PI had a lower survival than bumblebees fed pL and PL (Cox model, PI–pL: HR = 1.62; 95% CI 1.14–2.29; $p < 0.05$; PI–PL: HR = 2.12; 95% CI 1.46–3.06; $p < 0.001$). Bumblebees fed pL and PL had similar survival (Cox model, pL–PL: HR = 1.30; 95% CI 0.90–1.87; $p = 0.49$). Bumblebees fed PI in the absence of brood survived the least long (Figure 22.b), whereas bumblebees fed pL and N60% in the presence of brood survived the longest (Figure 22.a). Thus, overall, bumblebees fed PI died faster than bumblebees fed high-lipid diets (pL and PL).

Adult emergence rate

To explore the influence of diet on larval development, we compared the emergence rate of new adults in the different test conditions. In the no-choice experiment, the rate of emergence was higher in microcolonies fed PI than in microcolonies fed pL (Figure 22.c, Cox model, PI–pL: HR = 3.07; 95% CI 1.69–5.58; $p < 0.01$). There was no difference between all the other conditions (Cox model, pL–Choice: HR = 0.52; 95% CI 0.31–0.89; $p = 0.07$; PI–Choice: HR = 1.60; 95% CI 0.97–2.64; $p = 0.24$; PL–Choice: HR = 0.96; 95% CI 0.57–1.62; $p = 0.99$; PL–pL: HR = 1.85; 95% CI 1.02–3.34; $p = 0.17$; PL–PI: HR = 0.60; 95% CI 0.34–1.06; $p = 0.29$). Therefore, PI seemed to favour larval development. The type of liquid diet had no effect on adult emergence rate (Cox model, N60–N10: HR = 0.99; 95% CI 0.68–1.45; $p = 0.97$; post hoc Tukey HSD from Cox model). We did not observe male larvae produced by workers over the course of the observations.

Lipid body content

To assess the effect of diet on physiology, we measured the lipid body content of bumblebees in the different test conditions. The type of solid diet influenced lipid body content (Figure 22.d; ANOVA, solid diet: $F_{3,136} = 4.91$; $p < 0.05$), whereas neither liquid diet type, nor the presence of brood in the nest, or their interactions had a significant effect (ANOVA, liquid diet: $F_{1,136} = 0.68$; brood: $F_{1,136} = 1.09$, $p = 0.30$; all interactions: $p > 0.05$). Post hoc comparisons did not reveal significant difference between test conditions (post hoc Tukey HSD, PI–pL: $p = 0.96$; PL–pL: $p = 0.60$; Choice–pL: $p = 0.85$; PL–PI: $p = 0.47$; Choice–PI: $p = 1.00$; Choice–PL: $p = 0.16$). However, bumblebees in the choice experiment were significantly fatter than bumblebees fed high-protein (PI and PL) in the no-choice experiment (Figure 22.d, post hoc Tukey HSD, Choice PI: $p < 0.01$; Choice–PL: $p < 0.05$), indicating that diet influenced lipid body content.

Discussion

We used a 3D nutritional geometry design to study how bumblebees regulate their collection of protein, carbohydrates, and lipids from artificial liquid and solid diets. Microcolonies given a choice between complementary diets self-selected foods to reach a nutrient collection target ratio of 71% proteins, 6% carbohydrates, and 23% lipids, irrespective of the presence of brood in the colony. However, when confined to an imbalanced diet, bumblebees either only regulated lipid collection or both lipid and protein collection simultaneously, depending on the presence of brood in the colony. This indicates that protein regulation is influenced by brood.

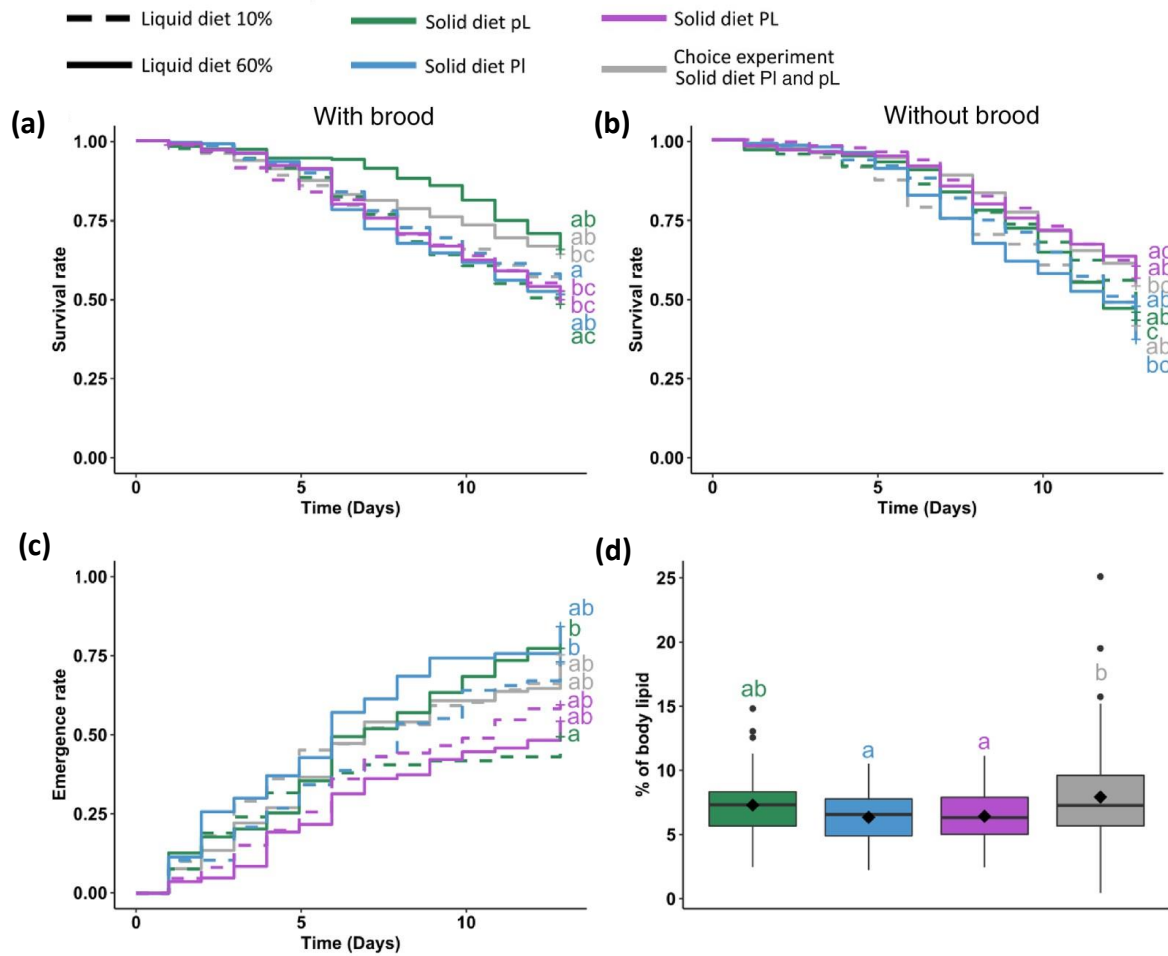


Figure 22: Fitness performances depending on brood presence and diet. Curves represent survival rate over 13 days for (a) microcolonies of bumblebees with brood and (c) microcolonies without brood. (c) Adult emergence rate. Curves represent emergence rate over 13 days for microcolonies with brood. Different letters above bars represent significant differences ($p < 0.05$, Tukey post hoc test after a Cox model). Eight to 10 microcolonies were used per test condition (see details about sample sizes in Table 5). (d) Lipid body content. Boxplot of the proportion of body content in bumblebees died at least 5 days after the start of the experiment. The central line is the median, the edges of the box are the 25th and 75th percentiles, the whiskers extend to the most extreme data points, outliers are represented by points, and diamonds represent means. Different letters above bars represent significant differences ($p < 0.05$, Tukey post hoc test after a mixed-effect ANOVA). About 5–18 bumblebees were analysed per test condition (see sample size details in Table 5).

In nature, bees must extract key nutrients from plant nectars and pollens. Several recent studies show how individual workers or small groups of workers self-compose their diets to balance their acquisition of carbohydrates and proteins (honeybees: Paoli et al., 2014a; bumblebees: Stabler et al., 2015), or proteins and lipids (bumblebees: Vaudo et al., 2016a,b) from liquid or solid artificial diets. Here, using both diet types, we demonstrate that variations of protein, lipids, and carbohydrates, simultaneously or independently, influence food collection by microcolonies. Our data validate this novel approach by demonstrating that bumblebees did not collect artificial diets randomly, were physiologically affected by the diets, and even stored diets in empty brood cells as they would do with natural nectars and pollens (Goulson, 2010).

In this study, we designed diets with extreme variation in nutrient ratio that cannot be considered as strictly equivalent to natural nectars and pollens. This was done to test the ability of bees to regulate their nutrient collection in the broadest possible nutrient space. Although this approach is useful to reveal qualitative effects of nutrition on the behaviour and physiology of bees, the amplitude of the effects observed may be different when bees feed on more natural diets. Previous studies showed how bee nutrient intake depend on the type of diet proposed (Stabler et al., 2015), suggesting that the nutrient collection target ratio measured in our study could strongly differ from that of bumblebees in the field. The strong mortality observed in our experiments might be due to the extreme nature of the artificial diets used. Future studies should therefore attempt to refine diet compositions to simulate natural nutritional environments to bees.

Improvements could be done, for instance, by using amino acid composition of proteins closer to that found in natural pollens (Vanderplanck et al., 2014; Moerman et al., 2016), adding micronutrients beneficial to insects (Cohen, 2015), and using sterols that are more likely to be digestible for bumblebees and abundant in natural pollen such as β -sitosterol or d5-avenasterol (Vanderplanck et al., 2014).

Exposing microcolonies to different diets showed that bumblebee colonies actively regulate their intake of proteins, carbohydrates, and lipids, thereby confirming previous observations with simpler, 2D nutritional designs (Vaudo et al., 2016a,b). Differences in artificial diets and methodological approaches likely explain differences between the intake ratios we measured and those reported in previous studies (e.g., Vaudo et al., 2016b: 1.4% proteins, 98.5% carbohydrates, and 0.1% lipids). First, we varied the ratios of 3 nutrients in foods simultaneously. Second, we used microcolonies with nest materials, whereas previous studies used individual workers or small groups of workers without brood (honeybees: Altaye et al., 2010; Pirk et al., 2010; Paoli et al., 2014a; bumblebees: Stabler et al., 2015; Vaudo et al., 2016b). Third, we provided liquid sources of carbohydrates and solid sources of proteins and lipids to bees that they could store in empty brood cells, an approach that only permitted to measure food collection, not real consumption by bees. Note, however, that the fact that the body lipid composition of bumblebees varied with pollen type demonstrates that colonies consumed part of (if not all) the pollen collected. Future studies using automated tracking to quantify food collection and ingestion (including water) by individuals (Greenwald et al., 2015), and food storage in cells (Colin et al., 2018) will help understand how bumblebees balance their diet from nectar and pollen resources, and whether this varies between individuals, for instance, among members of different castes.

The presence of brood in the colony did not influence the ratio of nutrients collected by bumblebees offered complementary diets in contrast to what was observed in ants (Dussutour and Simpson, 2009). This result is consistent with previous observations on another bumblebee species (*B. impatiens*) where foragers reached a similar protein to lipid intake ratio in a full-size colony or in a cage isolated from brood, suggesting that the nutritional needs of adult bumblebees closely match those of the larvae (Vaudo et al., 2016a). Nonetheless, we found that the presence of brood influenced food collection when colonies were confined on imbalanced diets. In these extreme conditions, bumblebees behaved as if they regulate lipids collection to reach a fixer target value of

9.09 mg/bee/day (7.97-10.21 95% CI, N=101 colonies). By contrast, protein regulation was only evident in the presence of brood.

Further experiments are needed to confirm this result in natural colonies containing all brood stages, workers, and a queen. For instance, the overcollection of protein observed in microcolonies without brood could be caused by the absence of queen pheromones that prevents the reproduction of workers and the development of ovaries that requires protein (Tasei and Aupinel, 2008b; Goulson, 2010). It would be interesting to use colonies in which the age of workers and the development stage of larvae are controlled, since the nutritional needs of bumblebees are likely to be age or caste dependent, as this is the case in honeybees (Schmickl and Crailsheim, 2002; Paoli et al., 2014a).

In our experiments, nectar collection was very low in comparison to pollen collection, suggesting that bumblebees did not give an equal importance to carbohydrates, proteins, and lipids. Since carbohydrates are a primary source of energy for flight (Brodschneider and Crailsheim, 2010), energy costs should vary depending on the distance of the food sources to the nest. Previous studies show that nectar intake by bees increases with the distance to food location (honeybees: Núñez and Giurfa, 1996) and those foragers integrate nectar intake rate in their spatial decisions (bumblebees: Lihoreau et al., 2011). In our experiment, energy needs were modest as the nest boxes were relatively small and bees did not need to fly to collect food. Manipulating the distance of pollen and nectar sources from the colony would allow us to explore the potential trade-off made by foragers between collecting carbohydrates for their own energy demands or collecting proteins and lipids for the colony needs.

So, why regulate lipid and protein, and not carbohydrates as previously seen in ants (Dussutour and Simpson, 2008; 2009; Cook et al., 2010; Bazazi et al., 2016)? Lipids are key for bee development, both at the larval and adult stages. Lipid metabolism participates in molting hormone production (e.g., ecdysone; Canavoso et al., 2001), larval growth and development (Vanderplanck et al., 2014), production of cuticular hydrocarbons and wax, behavioral maturation in adults (through the reduction in lipid

stores), diapause, development of glands that produce brood food (Canavoso et al., 2001; Toth and Robinson, 2005; Fliszkiewicz and Wilkaniec, 2007), and learning (Arien et al., 2015; 2018). In addition, an excess of polyunsaturated fatty acids may lead to lipid peroxidation and cell damage, which could explain the difference in queen and worker lifespan, where queens are protected from peroxidation and live longer than workers (Haddad et al., 2007).

Protein is also vital for larval development (Wright et al., 2018), as confirmed by the highest emergence rates in microcolonies given high-protein diets. However, high-protein diets (or diets rich in amino-acids) also reduce lifespan in many insects (e.g., drosophila: Lee et al., 2008a; field crickets: Maklakov et al., 2008; ants: Dussutour and Simpson, 2012; termites: Poissonnier et al., 2018; honeybees: Altaye et al., 2010; Pirk et al., 2010; Archer et al., 2014; Paoli et al., 2014b); including bumblebees: Stabler et al., 2015). An excess of protein is believed to increase toxic nitrogen waste products and overstimulate nutrient-sensing pathways that regulate lifespan (such as the Target of Rapamycin (TOR) pathway) (reviewed in Le Couteur et al., 2016).

The fact that protein regulation was most evident in the presence of brood, as shown in ants (Dussutour and Simpson, 2009) suggests that protein balance is more critical for brood development and adult maturation.

Malnourished larvae develop malformations that impact lifespan, dry weight, protein content, and wing size (Brodschneider and Crailsheim, 2010). Bee colonies tend to terminate brood rearing rather than produce malnourished adults (Schmickl and Crailsheim, 2002), a result that may explain the difference we found in the adult emergence rates of colonies in extreme nutritional conditions of no-choice experiments. In bumblebees, information on pollen quality and its availability in the colony may be accessible to workers via empty brood cells (Dornhaus and Chittka, 2005) allowing the colony to make informed foraging decisions. Our results thus suggest that protein regulation is based on cues yielding information about the nutritional state of larvae, although this will need to be confirmed with further investigations.

Nutritional geometry studies have recently changed the views about the importance of malnutrition for bee health and population declines (Wright et al., 2018). Our approach using diets varying in their ratio of 3 macronutrients shows that bee nutritional decisions are a multidimensional process that depends on colony composition. Beyond informing about nutritionally adequate mixes of plants that may help maintain sustainable bee populations (Vaudo et al., 2015), the development of solid and liquid synthetic diets for bees offers the possibility for future explorations of the spatial dimension of nutritional decisions (Lihoreau et al., 2017), by studying how the foraging patterns of bees are affected by the heterogeneity of pollen and nectars available in flowers, and how this influences plant–pollinator interactions and the critical pollination service they provide.

[Jedi Master Yoda: How you get so big eating food of this kind?]

-- Star Wars: Episode V - The Empire Strikes Back (1980)



CHAPTER 3

HEAT STRESS MODIFIES
CARBOHYDRATES, LIPIDS AND PROTEINS
REGULATION BY BUMBLEBEES

CHAPTER 3: Heat stress modifies carbohydrates, lipids and proteins regulation by bumblebees

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Summary:

- Here we studied the effects of temperature variations on the nutritional behaviour of bumblebees, using artificial liquid diets varying in their ratios of proteins, carbohydrates, and lipids.
- We found that bees adapt their nutritional behaviours to environmental temperature.
- At optimal temperature for brood development, workers achieved a precise nutritional intake to optimize their reproductive development.
- At lower temperature, bees search only for carbohydrates to prioritize survival, and at higher temperature, they start to collect more water and/or lipid to face heat stress that increase bees' mortality.
- Identifying the nutritional needs and behavioural changes of bees across seasons and extreme climatic events is necessary to ameliorate conservative actions.

Key words: bumble bees; artificial diets; climate change; heat stress; malnutrition

Introduction

Consuming nutrients in an adequate amount and balance is a critical challenge to all animals. Nutritional ecology research shows animals have developed strategies to regulate their intake of multiple key nutrients by collecting complementary food sources (Simpson and Raubenheimer, 1993; 2012). In doing so, they often must trade-off between favouring some traits over others to maximize their overall fitness (Lee et al., 2008a; Maklakov et al., 2008; Morimoto and Lihoreau, 2019). Environmental conditions, such as temperature changes, can dramatically influence these nutritional trade-offs by affecting the availability of foods, their nutrient contents, as well as the metabolic and energetic requirements of animals to metabolize these resources (Clissold and Simpson, 2015; Cross et al., 2015). For instance, recent studies using designs of nutritional geometry (Simpson and Raubenheimer, 2012) showed that a performance trait such as survival can vary with both diet composition and temperature (e.g., caterpillars: Lee et al., 2015; grasshoppers: Schmitz et al., 2016; beetles: Rho and Lee, 2017; drosophila: Kim et al., 2020). However, while the relative influences of temperature and nutritional balance could vary between traits, their interaction remains largely unclear (Clissold and Simpson, 2015).

This interaction is particularly relevant to pollinators that suffer important declines partly due to climate change and malnutrition (Goulson et al., 2015; Soroye et al., 2020). Bees exclusively depend on flowers to fulfil their nutritional requirements: nectars are their primary source of carbohydrates and pollens provide them with proteins, lipids and other micronutrients (Nicolson, 2011). Bee foragers must regulate their collection of these nutrients to meet their own needs as well as those of their brood (which are different), and those of other adults in the case of social species (Lihoreau et al., 2018). Individual bees or small groups of bees were found to self-select food sources in the lab in order to achieve a specific diet optimising fitness traits (Altaye et al., 2010; Paoli et al., 2014a; Stabler et al., 2015; Vaudo et al., 2016a,b; Kraus et al., 2019a). In this way, bees regulate their intake of macronutrients obtained from nectar (Altaye et al., 2010: proteins and carbohydrates) and pollen (Vaudo et al., 2016a: protein and lipids), as well

as from artificial diets (Kraus et al., 2019a: proteins, carbohydrates and lipids). They can even discriminately select nectars based on the presence of single essential individual amino acids (Hendriksma and Shafir, 2016).

All these experiments so far have been conducted under stable laboratory conditions. However, temperature variations during the day and across seasons may dramatically affect these diet regulatory behaviours, and impact fitness traits such as survival (Paoli et al., 2014b; Vaudo et al., 2016b), reproduction and development (Tasei and Aupinel, 2008a; Moerman et al., 2016; 2017), immune response (Di Pasquale et al., 2013) and cognition (Arien et al., 2015; 2018; Muth et al., 2018). Indeed, as plant growth and photosynthesis are affected by heat and hydric stress (Pandey et al., 2015), the quantity and quality of nectars and pollens in flowers are negatively correlated with temperature (Descamps et al., 2018; 2021b), which in turn affects the behaviour of pollinators. Plants exposed to warm temperatures tend to produce less flowers, less nectar per flower, and less concentrated nectars, and are thus less frequently visited by bumblebees (Descamps et al., 2021a). Temperature elevation also influences the energy needs of bees. First, to maintain in-nest homeostasis (30°C: Gardner et al., 2007; Gürel and Gösterit, 2008; Nasir et al., 2019) workers produce heat at low temperatures or fan their wings at high temperatures (Heinrich, 2004; Stabentheiner et al., 2010; Kelemen and Dornhaus, 2018). Second, foragers need to maintain a high internal temperature (30 - 44°C) to be able to fly, which requires higher energy at low environmental temperatures (Heinrich, 1979).

In this chapter, we experimentally studied the effects of temperature variations on the nutritional behaviour of bumblebees, using artificial liquid diets varying in their ratios of proteins, carbohydrates, and lipids. We first determined the nutritional choice of micro-colonies when provided multiple complementary diets at 30°C, a temperature expected to be optimal for colony growth (Gürel and Gösterit, 2008; Nasir et al., 2019). Second, we repeated the experiment at lower (20°C) and higher (35°C) temperatures, to test the assumption that the nutritional choice would differ due to constraints imposed by nest thermoregulation. Third, we constrained micro-colonies on single diets at 30°C and at 20°C to measure performance indicators. We hypothesized that bumblebees would

adapt their nutrient intake to temperature, and that survival would require a sugar diet while egg-laying would need an optimal temperature and more proteins.

Material and Methods

Bumblebees

Experiments were conducted between June 2020 and August 2021. A total of 57 commercial colonies of *B. terrestris* (Koppert BV, Berkel en Rodenrijs, The Netherlands) were used. Each colony contained about 200 workers, brood and a queen. Colonies were maintained at 30°C and 50% relative humidity, under a 12:12h light:dark photocyclus. Room temperature was controlled every five minutes using a data-logger (Elitech, London, United Kingdom). Colonies were fed *ad libitum* with commercial sugar syrup (Koppert BV, Berkel en Rodenrijs, Netherlands). Access to the syrup was blocked for 6 days before the experiments.

Artificial diets

We designed artificial liquid diets using the approach described in Chapter 1.B (see Methods pages 45-46), composed of the same mix of proteins, lipids and carbohydrates described in Chapter 1.A (see Methods page 39). We used 28 diets varied in their ratios of protein, lipids, and carbohydrates (Table 7 and Figure 23.a), including those used in Chapter 1.B.

Table 7: Recipes of the 28 artificial diets used in nutrition and temperature experiment. Percentages of carbohydrates, lipids and proteins in each diet in calories. Amounts of carbohydrates, lipids, proteins, total nutrients, and water in grams per kilograms. Ratios of each macronutrient in relation to the two others in calories, and ratio of all nutrients in relation to water. Macronutrient mixtures are composed of the same ingredients in similar proportions as described in Chapter 1.A (see Methods page 39). Differences between mixtures and nutrients amounts were due to ashes or water in ingredients.

Diets	1	2	3	4	5	6	7
% Carbohydrate	90.00	75.83	75.83	61.66	61.66	61.66	47.50
% Lipid	5.00	5.00	19.16	5.00	19.16	33.33	5.00
% Protein	5.00	19.16	5.00	33.33	19.16	5.00	47.50
C in diet (g/kg)	142.50	120.06	120.06	97.63	97.63	97.63	75.20
L in diet (g/kg)	3.51	3.51	13.48	3.51	13.48	23.45	3.51
P in diet (g/kg)	7.91	30.34	7.91	52.77	30.34	7.91	75.20
Total Nutrient (g/kg)	153.93	153.93	141.47	153.93	141.47	129.01	153.93
Water (g/kg)	835.60	832.02	848.21	828.43	844.62	860.81	824.84
Ratio N : W	5.43 : 1	5.41 : 1	6 : 1	5.38 : 1	5.97 : 1	6.67 : 1	5.36 : 1
Ratio C : (L+P)	9 : 1	3.14 : 1	3.14 : 1	1.61 : 1	1.61 : 1	1.61 : 1	1 : 1.11
Ratio L : (C+P)	1 : 19	1 : 19	1 : 4.22	1 : 19	1 : 4.22	1 : 2	1 : 19
Ratio P : (C+L)	1 : 19	1 : 4.22	1 : 19	1 : 2	1 : 4.22	1 : 19	1 : 1.11
Carbohydrate Mix (g/kg)	144.15	121.46	121.46	98.77	98.77	98.77	76.08
Lipid Mix (g/kg)	3.55	3.55	13.64	3.55	13.64	23.73	3.55
Protein Mix (g/kg)	9.27	35.55	9.27	61.83	35.55	9.27	88.10

Diets	8	9	10	11	12	13	14
% Carbohydrate	47.50	47.50	47.50	33.33	33.33	33.33	33.33
% Lipid	19.16	33.33	47.50	55.00	19.16	33.33	47.50
% Protein	33.33	19.16	5.00	61.66	47.50	33.33	19.16
C in diet (g/kg)	75.20	75.2	75.20	52.77	52.77	52.77	52.77
L in diet (g/kg)	13.48	23.45	33.42	3.51	13.48	23.45	33.42
P in diet (g/kg)	52.77	30.34	7.91	97.63	75.20	52.77	30.34
Total Nutrient (g/kg)	141.47	129.01	116.55	153.93	141.47	129.01	116.55
Water (g/kg)	841.04	857.23	873.42	821.26	837.45	853.64	869.83
Ratio N : W	5.94 : 1	6.64 : 1	7.49 : 1	5.34 : 1	5.92 : 1	6.62 : 1	7.46 : 1
Ratio C : (L+P)	1 : 1.11	1 : 1.11	1 : 1.11	1 : 2	1 : 2	1 : 2	1 : 2
Ratio L : (C+P)	1 : 4.22	1 : 2	1 : 1.11	1 : 19	1 : 4.22	1 : 2	1 : 1.11
Ratio P : (C+L)	1 : 2	1 : 4.22	1 : 19	1.61 : 1	1 : 1.11	1 : 2	1 : 4.22
Carbohydrate Mix (g/kg)	76.08	76.08	76.08	53.39	53.39	53.39	53.39
Lipid Mix (g/kg)	13.64	23.73	33.81	3.55	13.64	23.73	33.81
Protein Mix (g/kg)	61.83	35.55	9.27	114.38	88.10	61.83	35.55

Table 7: Recipes of the 28 artificial diets used in nutrition and temperature experiment. Percentages of carbohydrates, lipids and proteins in each diet in calories. Amounts of carbohydrates, lipids, proteins, total nutrients, and water in grams per kilograms. Ratios of each macronutrient in relation to the two others in calories, and ratio of all nutrients in relation to water. Macronutrient mixtures are composed of the same ingredients in similar proportions as described in Chapter 1.A (see Methods page 39). Differences between mixtures and nutrients amounts were due to ashes or water in ingredients.

Diets	15	16	17	18	19	20	21
% Carbohydrate	33.33	19.16	19.16	19.16	19.16	19.16	19.16
% Lipid	61.66	5.00	19.16	33.33	47.50	61.66	75.83
% Protein	5.00	75.83	61.66	47.50	33.33	19.16	5.00
C in diet (g/kg)	52.77	30.34	30.34	30.34	30.34	30.34	30.34
L in diet (g/kg)	43.39	3.51	13.48	23.45	33.42	43.39	53.36
P in diet (g/kg)	7.91	120.06	97.63	75.20	52.77	30.34	7.91
Total Nutrient (g/kg)	104.08	153.93	141.47	129.01	116.55	104.08	91.62
Water (g/kg)	886.03	817.67	833.86	850.05	866.25	882.44	898.63
Ratio N : W	8.51 : 1	5.31 : 1	5.89 : 1	6.59 : 1	7.43 : 1	8.48 : 1	9.81 : 1
Ratio C : (L+P)	1 : 2	1 : 4.22	1 : 4.22	1 : 4.22	1 : 4.22	1 : 4.22	1 : 4.22
Ratio L : (C+P)	1.61 : 1	1 : 19	1 : 4.22	1 : 2	1 : 1.11	1.61 : 1	3.14 : 1
Ratio P : (C+L)	1 : 19	3.14 : 1	1.61 : 1	1 : 1.11	1 : 2	1 : 4.22	1 : 19
Carbohydrate Mix (g/kg)	53.39	30.70	30.70	30.70	30.70	30.70	30.70
Lipid Mix (g/kg)	43.90	3.55	13.64	23.73	33.81	43.90	53.98
Protein Mix (g/kg)	9.27	140.66	114.38	88.10	61.83	35.55	9.27
Diets	22	23	24	25	26	27	28
% Carbohydrate	5.00	5.00	5.00	5.00	5.00	5.00	5.00
% Lipid	5.00	19.16	33.33	47.50	61.66	75.83	90.00
% Protein	90.00	75.83	61.66	47.50	33.33	19.16	5.00
C in diet (g/kg)	7.91	7.91	7.91	7.91	7.91	7.91	7.91
L in diet (g/kg)	3.51	13.48	23.45	33.42	43.39	53.36	63.33
P in diet (g/kg)	142.5	120.06	97.63	75.20	52.77	30.34	7.91
Total Nutrient (g/kg)	153.93	141.47	129.01	116.55	104.08	91.62	79.16
Water (g/kg)	814.08	830.27	846.47	862.66	878.85	895.05	911.24
Ratio N : W	5.29 : 1	5.87 : 1	6.56 : 1	7.4 : 1	8.44 : 1	9.77 : 1	11.51 : 1
Ratio C : (L+P)	1 : 19	1 : 19	1 : 19	1 : 19	1 : 19	1 : 19	1 : 19
Ratio L : (C+P)	1 : 19	1 : 4.22	1 : 2	1 : 1.11	1.61 : 1	3.14 : 1	9 : 1
Ratio P : (C+L)	9 : 1	3.14 : 1	1.61 : 1	1 : 1.11	1 : 2	1 : 4.22	1 : 19
Carbohydrate Mix (g/kg)	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Lipid Mix (g/kg)	3.55	13.64	23.73	33.81	43.90	53.98	64.07
Protein Mix (g/kg)	166.94	140.66	114.38	88.10	61.83	35.55	9.27

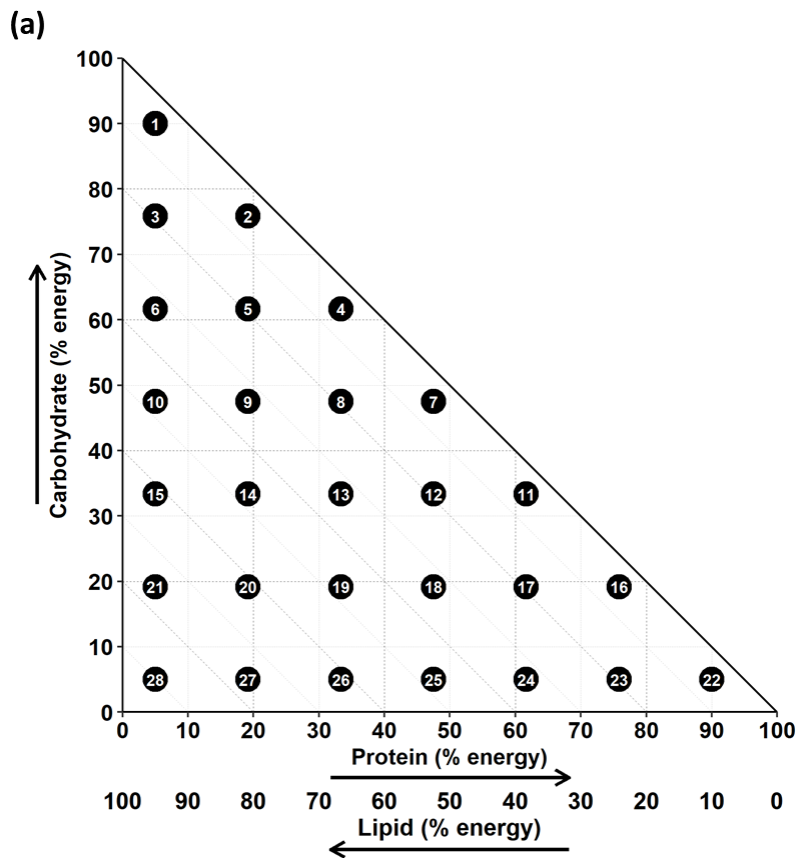
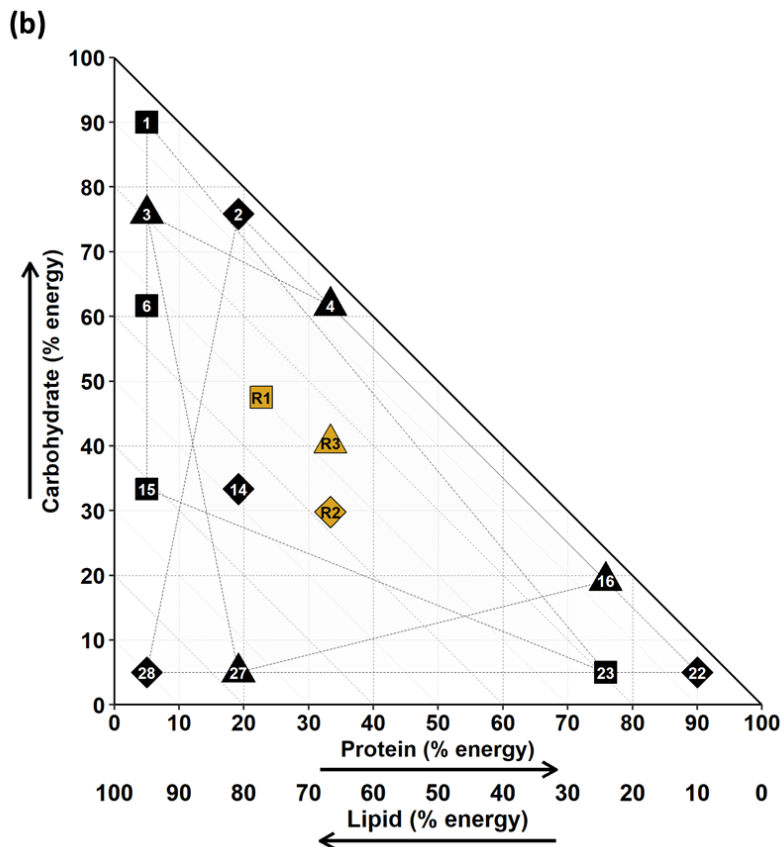


Figure 23: 3D nutrient space used in nutrition and temperature experiment. (a) All 28 artificial diets used (numbered dots); (b) the three choice configurations (1-3) with their respective four diets (numbered squares, diamonds or triangles) and corresponding random nutrient intake (yellow squares, diamonds or triangles) (similar approach as in Chapter 1.B).



Behavioural assay

We ran experiments on groups of adult workers. Each mother colony was divided in 6 to 12 micro-colonies, containing 20 workers and about 77.98g (± 10.49 CI95) of empty brood cells from the mother colony, which were placed in the peripheral chambers of a homemade designed dodecagonal arena (described in Chapter 1.B, Figure 9, page 47). The colony queen was placed in the central chamber containing brood cells and fed commercial sugar syrup (Koppert BV, Berkel en Rodenrijs, Netherlands).

Micro-colonies were maintained at 50% relative humidity, under a 12:12h light:dark photocycle, during 14 days. Room temperature was measured every 5 minutes using a data logger (Elitech, London, United Kingdom) and was in the range of 0.01°C of the desired temperature. We measured several performance traits: diet collection, workers survival, adult relative weight and egg-laying. Artificial diets were provided every day in a drilled 5ml Eppendorf vial with a new cup to collect leakage. Each vial and cup was weighed before introduction and after removal from food chambers, using a precision balance of 1mg (ME103T, Mettler Toledo, Greifensee, Suisse). Time between each renewal was measured. Potential food leakage was measured by weighing each diet in empty micro-colonies and was accounted for in the measure of food collection (see calculation in section below). Dead bees were counted, removed and frozen every day. Empty brood cells originating from the mother colony were placed in each micro-colony. Brood cells were dissected at the end to count the number of eggs, larvae and pupae.

Only eggs and larvae were counted as brood of the microcolony, because any pupae must have been produced by the mother colony (this takes about two weeks: Goulson 2010) and undetected when setting up the micro-colony.

We ran two types of experiments: choice and no-choice experiments. The choice experiments were designed to assess the nutrient intake target of bees (see concept in General Intro, pages 15-16), and were run at three temperature regimes: 20°C, 30°C and 35°C. Bumblebees of a given microcolony were given a choice between four complementary diets, out of 3 possible configurations (C1: 1, 6, 15, 23; C2: 2, 14, 22, 28; C3: 3, 4, 16, 27) (Figure 23.b). Using three combinations of pairs of diets allowed us to

discriminate between random choices (which would systematically correspond to the barycentre of each combination in the 3D nutrient space: Figure 23.b). The four diets were provided in drilled 5ml Eppendorf vials used as feeders, containing 4.36g (\pm 0.01 CI95).

The no-choice experiments were designed to assess consequences of different nutrient intakes on fitness, and to identify potential rules of compromise target used by bees (see concept in General Intro, pages 15-16). These experiments were run at two temperature regimes: 20°C and 30°C (at 35°C, mortality was too high to provide enough data: 50% at 3 days). Bumblebees were provided two gravity feeders of 6.25g (\pm 0.05 CI95) of the same diet. All 28 diets were used at 30°C, whereas only the 10 diets promoting higher survival at 30°C were used at 20°C to provide enough data. Some micro-colonies were only fed on water at 20°C or 30°C to serve as controls for survival, weight and egg-laying comparison. No-choice experiments were replicated eight times for each diet and temperature, but during one replicate at 30°C, an accidental decrease in room temperature led to keep only data before day 7 (half of the experiment).

Measure of food collection

We measured the average total amounts of diet and of each specific nutrient collected per microcolony each day following the methodology described in Chapter 1.B (see Methods page 49), and then divided these numbers by the number of bees still alive on each day to estimate individual value.

Body size and weight

We measured the body size and weight of every bee to control for potential size effects using thorax width and dry weight as described in Chapter 1.A. (see Methods page 41).

Statistical analysis

Data are presented in the text as median with the limits of 95% confidence intervals into brackets. All statistical analyses were performed in R 4.1.2 (R Core Team, 2021). For the choice experiments, tests were performed with groups of choice configuration, temperature, and their interaction. We used Kruskal-Wallis tests to analyse the effect of groups on the total amount of food and the different nutrient ratios collected. For all experiments, we used Kruskal-Wallis tests to analyse the effect of groups on the relative dry weight (in mg) for a median bee (of 4.5mm thorax width) and the brood size (number eggs and larvae) found at the end of experiments. We ran post hoc pairwise comparisons using Dunn tests with Bonferroni correction. We performed survival analyses using Cox Proportional-Hazards Models on groups in choice and no-choice experiments, followed by pairwise comparisons using Hazard Ratios.

Results

Choice experiment

Bees collected more diet and water as temperature increased

We tested 16 micro-colonies per choice configuration at 35°C, and 8 at 20°C and 30°C. Choice configurations did not influence the individual amount of collected food (Kruskal-Wallis: $H_{(2)} = 1.19$, $p = 0.55$). However temperature had a significant effect in interaction with choice configurations (Kruskal-Wallis: temperature: $H_{(2)} = 71.9$, $p < 0.001$; temperature x choice configuration: $H_{(8)} = 76.34$, $p < 0.001$): bumblebees collected more food as temperature increased, from 65.05 mg/bee/day [52.41;95.8] at 20°C to 166.43 mg/bee/day [136.98;238.9] at 30°C, and up to 467.29 mg/bee/day [321.67;647.42] at 35°C (Figure 24.a).

The provided diets were the only source of water in our conditions, but the ratio between the nutrients and water (N:W) varied between each diet. The ratio in the diet collected by each individual on average was not influenced by the diet choice

configuration (Kruskal-Wallis: $H_{(2)} = 4.84$, $p = 0.09$). However, it was significantly affected by room temperature and its interaction with choice configurations (Kruskal-Wallis: temperature: $H_{(2)} = 47.95$, $p < 0.001$; temperature x choice configuration: $H_{(8)} = 71.17$, $p < 0.001$): bumblebees collected more water with increasing of temperatures (Figure 2.b).

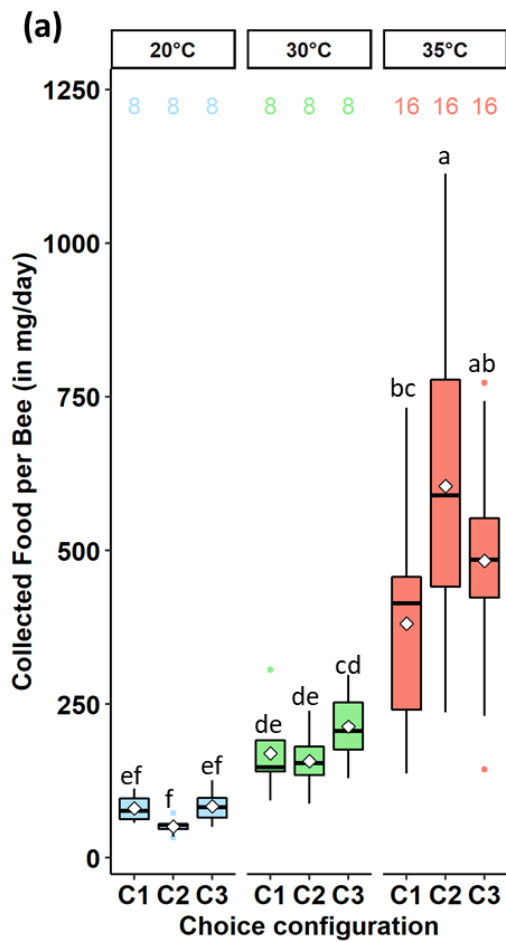


Figure 24: Bumblebees' food and water collection. (a) Boxplot of food collected per bee (in mg/day) at the end of the choice experiment, for each temperature and choice configurations. The central line is the median, the edges of the box are the 25th and 75th percentiles, the whiskers extend to the most extreme data points not without considering outliers, which are represented by points.

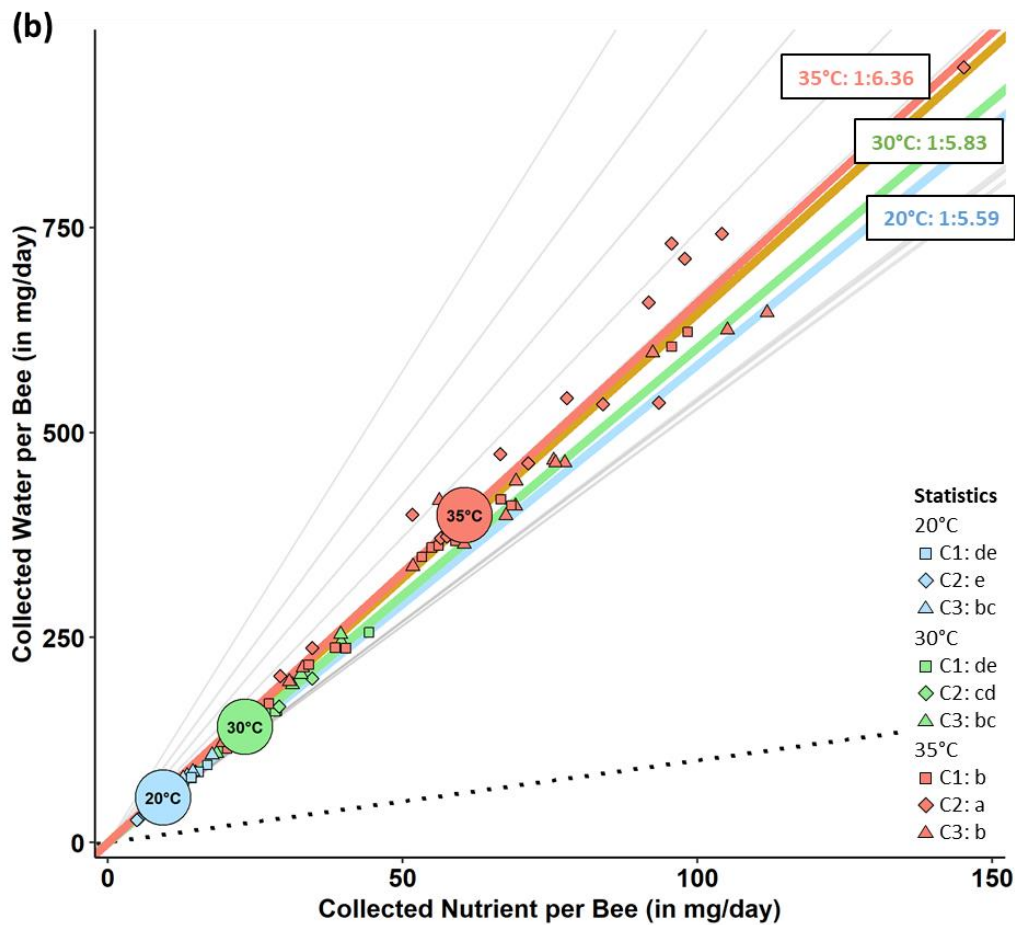


Figure 24: Bumblebees' food and water collection. (b) 2D nutritional geometric framework representing the amount of water and total nutrients collected. The dotted polygons correspond to the three choice configurations with their respective four diets (numbered square, diamond or triangle). Coloured squares, diamonds and triangles correspond to total diet collected by each micro-colony, while coloured circles are the median obtained at each temperature. Grey lines: ratio of each diet; yellow line: ratio expected from random choice; dotted line: 1:1 ratio; coloured lines: median ratios obtained in experiments with their value indicated. Different letters stand for significant differences.

Temperature influenced the nutritional behaviour of bees

Both the choice configuration and temperature had an effect on the C:(L+P), L:(C+P) and P:(C+L) ratios in the diet collected (Kruskal-Wallis: $H_{(2)} > 15$ and $p < 0.001$ in 8 cases), with the exception of choice configuration on the ratio L:(C+P) (Kruskal-Wallis: $H_{(2)} = 5.56$, $p = 0.06$). Besides, their interaction affected the three ratios overall (Kruskal-Wallis: $H_{(8)} > 15$ and $p < 0.001$ in all 3 cases). However, at 30°C the C:(L+P) and P:(C+L) ratios were similar across configurations, while all three ratios differed at 20°C and 35°C (Table 8). Therefore, bumblebees converged to a similar (non-random) nutritional intake

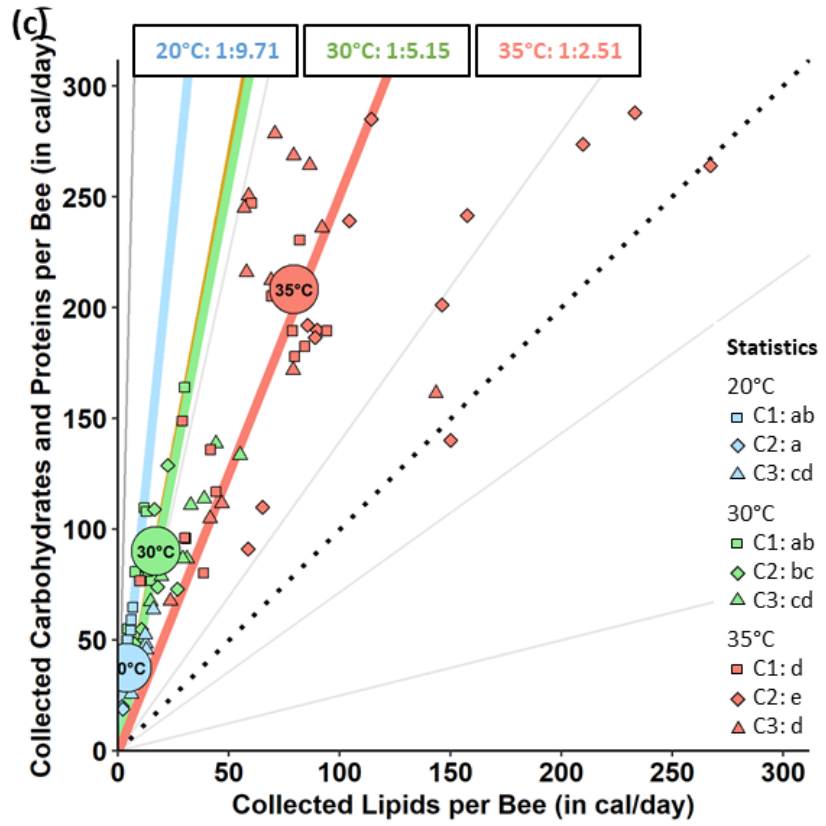
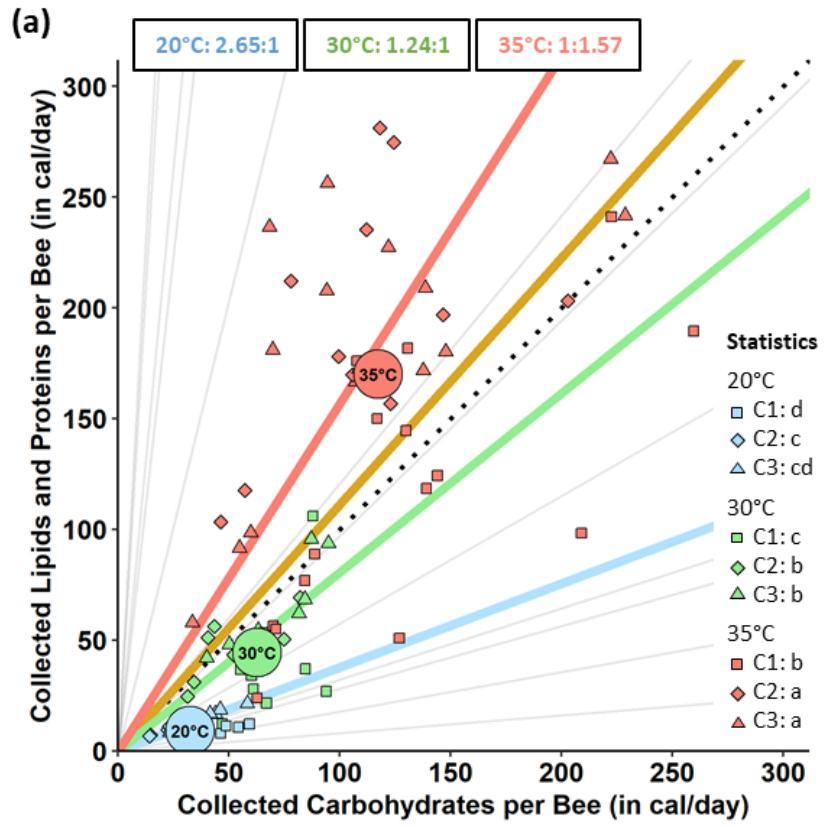
irrespective of choice configuration at 30°C, while at 20°C and 35°C they reached different nutrient intakes in the different choice configurations (Figure 25).

Low and high temperatures drove the nutrient balance toward carbohydrates and lipids, respectively

At the optimal temperature of 30°C, the average composition of the diet collected was of 25:59:16 P:C:L (% energy) (Figure 25.d). By contrast, lowering the temperature increased the proportion of carbohydrates (C:(L+P) from 1.24:1 to 2.65:1) and decreased that of proteins (P:(C+L) from 1:3.16 to 1:11.53). However, bees maintained a lipid ratio of 1:5.89 L:(C+P). Indeed, as already mentioned bumblebees collected less food at 20°C, but the drop was more brutal for proteins and lipids (divided by respectively 5.46 and 4.15) than for carbohydrates (divided by 1.93). On the other hand, shifting to 35°C increased the lipid ratio (L:(C+P): from 1:5.15 to 1:2.51 (Table 8b and Figure 25.b), as the increased collection was more pronounced for lipids (4.65 times more) than for proteins and carbohydrates (resp. 3.33 and 1.87 times more).

It thus appears that bumblebees maintained a specific lipid ratio while varying the balance between proteins and carbohydrates as temperature decreased, while the higher temperature shifted the general balance towards a more lipidic diet.

Daily collection per bee was of 62.78 cal of carbohydrates [43.38;84.68], 17.06 cal of lipids [10.69;31.11] and 27.28 cal of proteins [17.21;36.37] at 30°C.



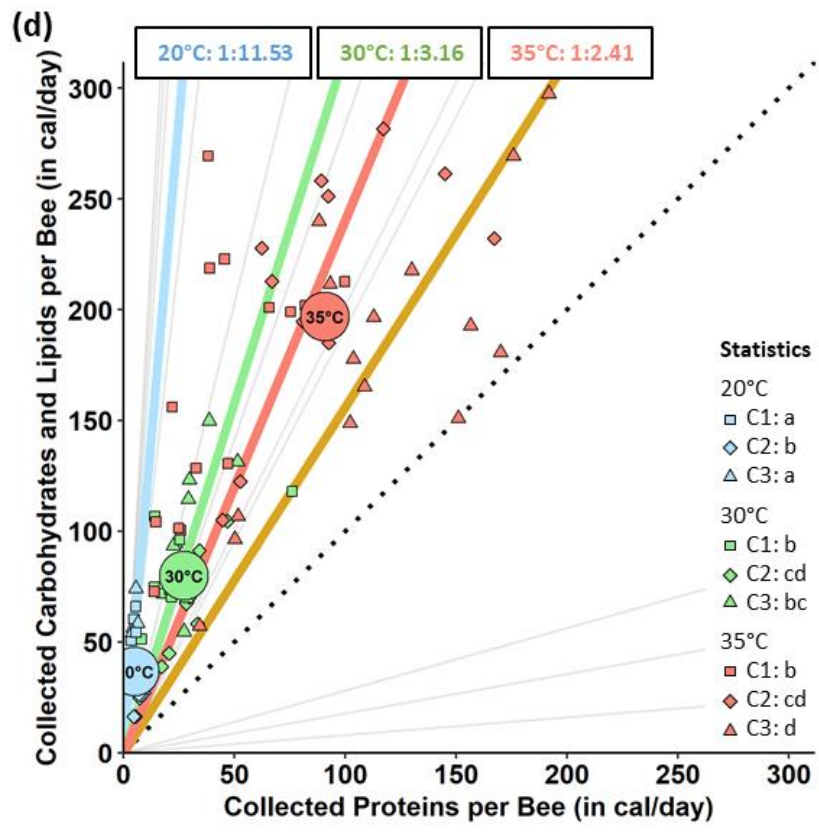
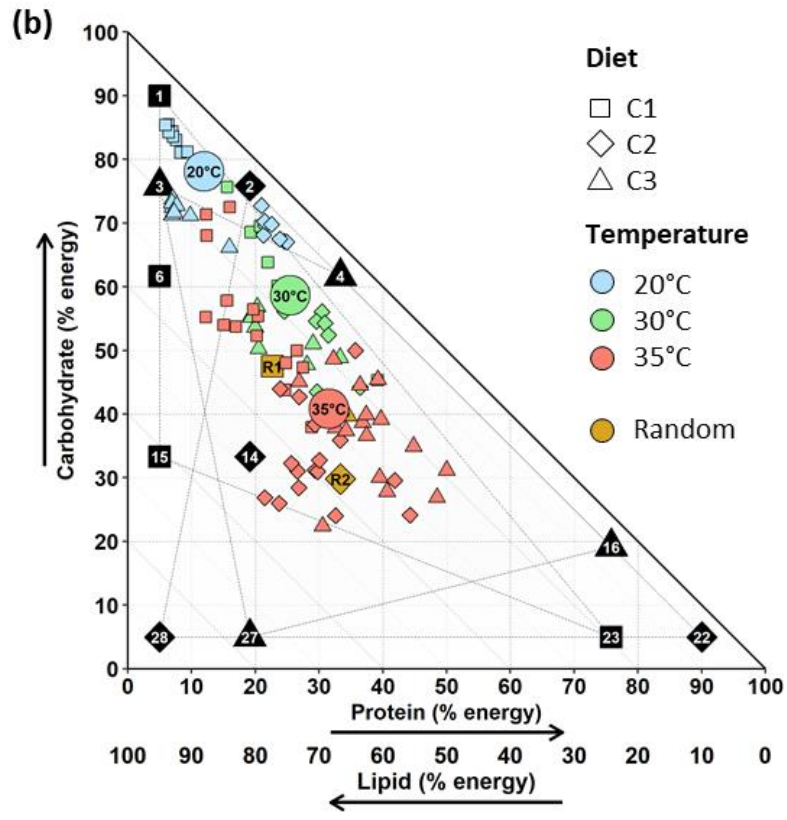


Figure 25: Nutritional geometric framework for temperature effect on choice experiments.

Nutritional geometric frameworks representing the respective amounts of collected nutrients in two dimensions (a,c,d) and a percentage of collected nutrients in three dimensions (b). (a) carbohydrates vs. lipids and proteins, (c) lipids vs. carbohydrates and proteins, (d) carbohydrates vs. lipids and proteins. Grey lines: ratio in each diet; yellow line: ratio expected for a random choice; coloured lines: median ratio obtained for each group; dotted line: 1:1 ratio. (b) Simultaneous representation of proportions of all nutrients in three dimensions, where the dotted polygons are the three choice configurations with their respective four diets (numbered square, diamond or triangle) and corresponding random points (yellow square, diamond or triangle). In all panels, coloured squares, diamonds or triangles indicate the proportions collected by each individual at the end of the experiment, while coloured circles indicate the median for each group (20°C, 30°C, 35°C). Different letters stand for significant differences.

Table 8: Paired comparisons of nutrient ratios were done confronting size effect with 95% confidence intervals generated by bootstraps. Choice configurations were compared in each temperature condition for ratios of **(a)** carbohydrates, **(b)** lipids and **(c)** proteins over the two other nutrients, in the collected diet. Comparison between temperatures (after pooling choice configurations) also provided. Significant differences are in red (*: p<0.05).

a. Carbohydrates ratio C:(L+P)		
	20°C_C1	20°C_C2
20°C_C2	0.26 [0.17;0.32] *	
20°C_C3	0.2 [0.13;0.29] *	-0.06 [-0.12;0.06]
30°C_C1		
	30°C_C1	30°C_C2
30°C_C2	0.39 [-0.21;0.99]	
30°C_C3	0.46 [-0.15;0.76]	0.07 [-0.5;0.32]
35°C_C1		
	35°C_C1	35°C_C2
35°C_C2	1.36 [0.19;2.25] *	
35°C_C3	0.8 [-0.05;1.99]	-0.56 [-1.61;1.12]
20°C		
	20°C	30°C
30°C	0.43 [0.04;0.87] *	
35°C	1.19 [0.53;2.11] *	0.76 [-0.06;1.79]
b. Lipids ratio L:(C+P)		
	20°C_C1	20°C_C2
20°C_C2	1.27 [-1.2;4.45]	
20°C_C3	-5.7 [-6.93;-4.65] *	-6.97 [-9.79;-5.04] *
30°C_C1		
	30°C_C1	30°C_C2
30°C_C2	-2.58 [-7.2;0.95]	
30°C_C3	-4.77 [-7.96;-1.43] *	-2.19 [-3.71;0.57]
35°C_C1		
	35°C_C1	35°C_C2
35°C_C2	-1.17 [-2.87;0.1]	
35°C_C3	0.29 [-1.78;2.03]	1.45 [0.03;3] *
20°C		
	20°C	30°C
30°C	-4.56 [-7.99;3.65]	
35°C	-7.2 [-9.19;-0.61] *	-2.64 [-5.97;0.51]
c. Proteins ratio P:(C+L)		
	20°C_C1	20°C_C2
20°C_C2	-9.64 [-12.22;-7.15] *	
20°C_C3	-0.19 [-8.03;2.55]	9.45 [3.5;10.39] *
30°C_C1		
	30°C_C1	30°C_C2
30°C_C2	-1.67 [-4.99;0.18]	
30°C_C3	-0.1 [-4.78;1.61]	1.58 [-0.5;2.15]
35°C_C1		
	35°C_C1	35°C_C2
35°C_C2	-1.61 [-4.55;0.48]	
35°C_C3	-2.34 [-5.09;-0.6] *	-0.73 [-1.92;0.3]
20°C		
	20°C	30°C
30°C	-8.37 [-11.16;0.54]	
35°C	-9.12 [-11.72;-0.09] *	-0.76 [-2.51;1.32]

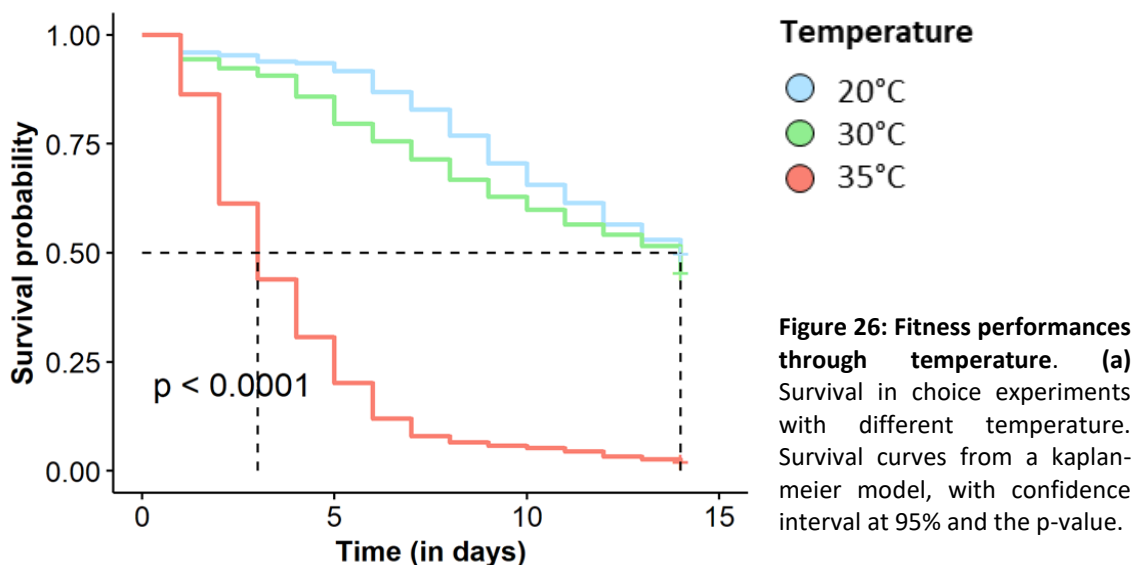
Heat stress was detrimental for workers survival, relative weight and egg-laying

Besides food and diet collection, three performances traits were measured during choice experiments to assess their sensitivity to temperature variations worker survival, relative weight and egg-laying.

The survival probability of bumblebees was not influenced by the choice configuration (Cox Model: Deviance analysis: $p = 0.23$), but it was affected by temperature ($p < 0.001$) as well as by its interaction with choice configuration ($p < 0.01$). At 35°C , they had a 8.5 times greater probability of death than at 20°C or 30°C (Cox Model: estimate = $2.1 (\pm 0.22)$, $z = 9.9$, $p < 0.001$; Figure 26.a).

The relative dry weight (in mg) for a median bee (of 4.5mm thorax width) was not influenced by choice configuration (Kruskal-Wallis: $H_{(2)} = 5.39$, $p = 0.07$), but temperature and mortality had effects on it, as well as the interactions between those three factors ($H > 15$ and $p < 0.001$ in 5 cases). Those bees still alive at the end of the experiment weighed 40.77 mg [-12;94.92] more than those found dead, as expected from a continuous intake during the experiment duration. In addition, bees were 1.5 times lighter at 35°C than at 20°C or 30°C (Figure 26.b).

The final brood size was influenced by the temperature and its interaction with choice configuration (Kruskal-Wallis: $H > 15$ and $p < 0.001$), but not by configuration alone ($H_{(2)} = 0.04$, $p = 0.98$). Bees tested laid 0.43 eggs per day [0;0.89] at 30°C , but almost none at 20°C or 35°C (Figure 26.c).



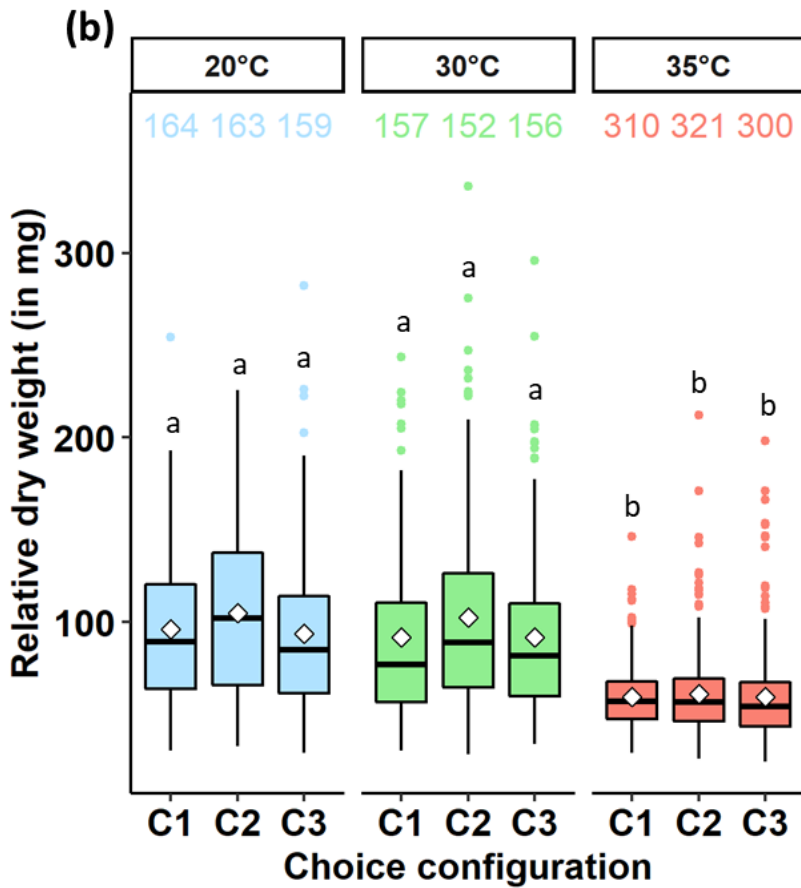
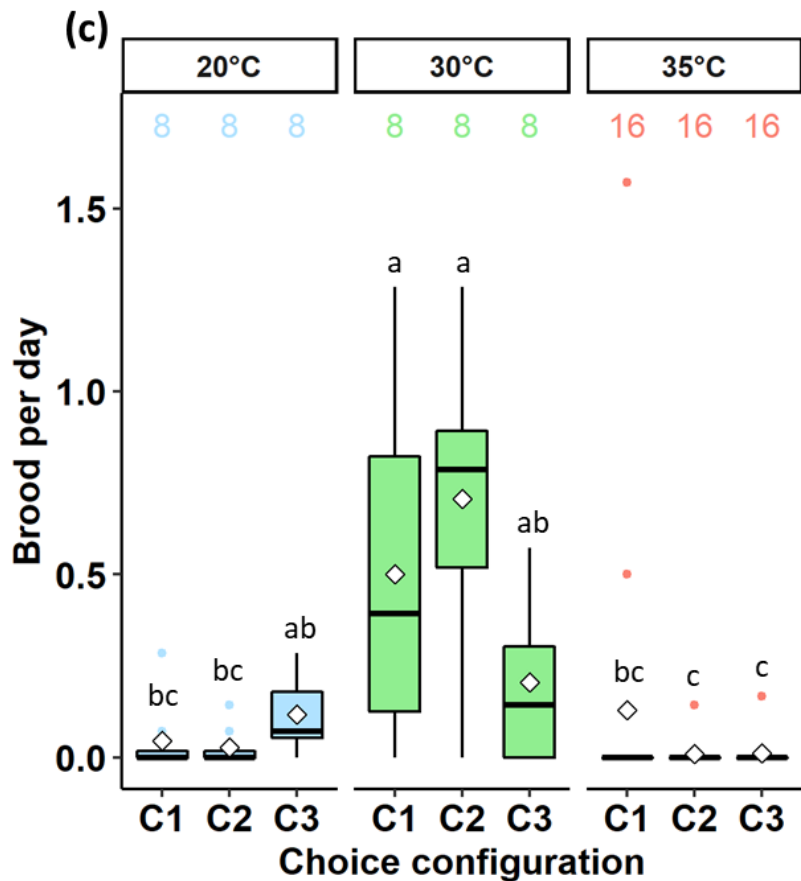


Figure 26: Fitness performances through temperature. (a) Survival in choice experiments with different temperature. Survival curves from a kaplan-meier model, with confidence interval at 95% and the p-value. (b) Boxplot of relative dry weight (in mg) for a median bee (of 4.5mm thorax width). (c) Boxplot of total brood found at the end of choice experiment. The central line is the median, the edges of the box are the 25th and 75th percentiles, the whiskers extend to the most extreme data points not considered outliers, outliers are represented by points. Different letters stand for significant difference.



No-choice experiment

Bumblebees prioritized workers their own weight at 30°C, while favouring survival at 20°C

Workers' survival, relative weight and egg-laying were measured in the absence of any choice, to identify which of these performance traits were prioritized, at 30°C or 20°C.

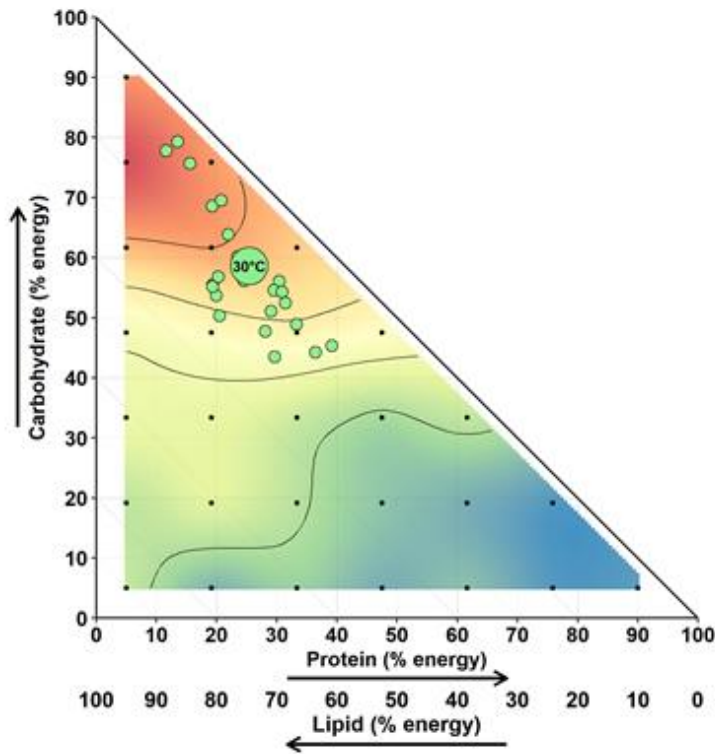
Survival probability was influenced by diet (Cox Model: Deviance analysis: $p < 0.001$). For both temperatures, the best survival performances were found for those diets richer in carbohydrates (mostly diets 2 and 3) while the worst ones were found for diets richer in proteins (Figure 27.a-b).

The relative dry weight (in mg) for a median bee (of 4.5mm thorax width) was also influenced by diet, at both 30°C (Kruskal-Wallis: $H_{(28)} = 125.99$, $p < 0.001$) and 20°C (Kruskal-Wallis: $H_{(10)} = 41.69$, $p < 0.001$). Larger bees were fed with diet rich in diet in carbohydrates (mostly diet 2 and 5) (Figure 27.c-d).

By contrast, brood size remained unaffected by diet at either temperature (30°C: $H_{(28)} = 16.91$, $p = 0.95$; 20°C: $H_{(10)} = 15.48$, $p = 0.12$) (Figure 27.e-f).

When comparing these performance maps and the previous nutritional balance achieved at each temperature in the choice experiment (Figure 27), we observe that, at 30°C, bumblebees collected available diets in such proportions that they could achieve a macronutrient ratio that best fits with a gain in individual weight, whereas at 20°C they collected richer carbohydrates diet to maximize their survival.

(a) 30°C Survival



(b) 20°C Survival

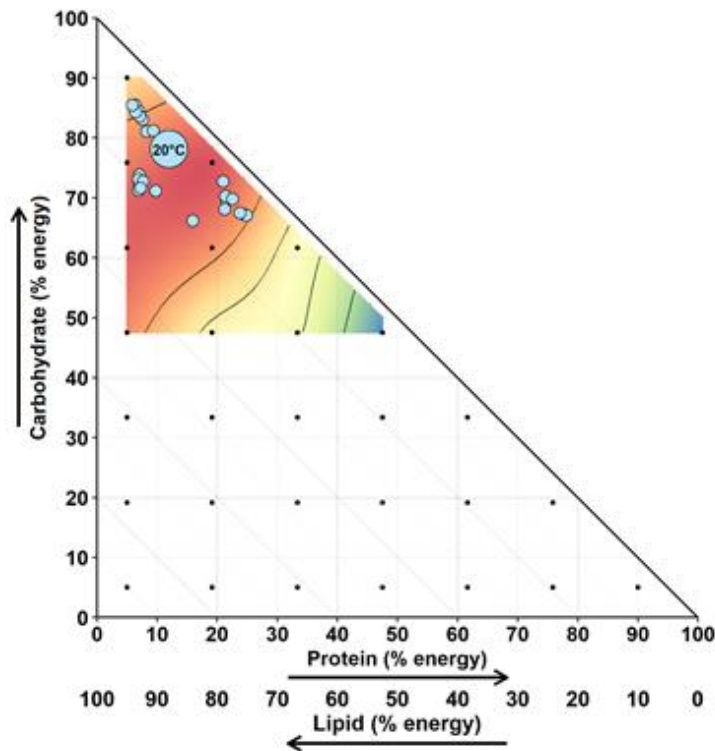
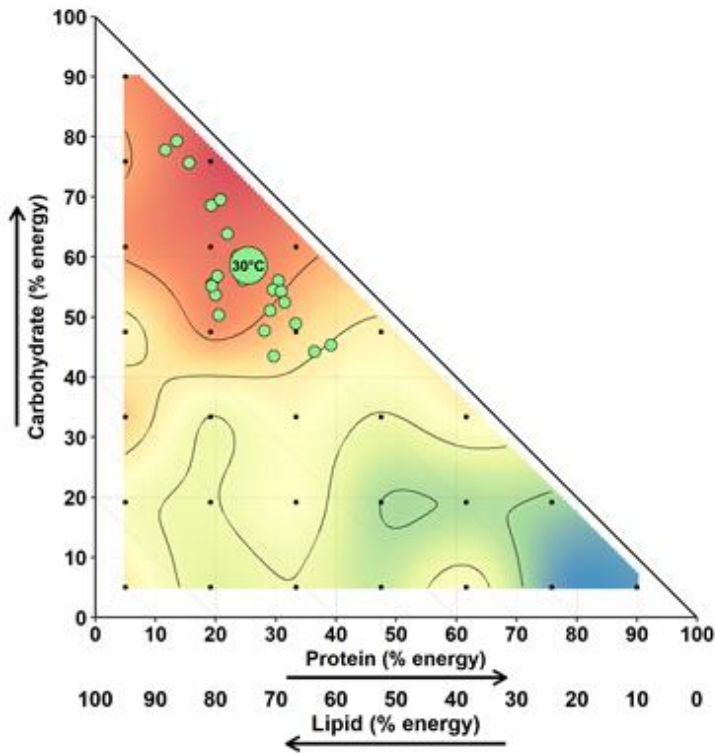


Figure 27: Performance traits in no-choice situations and comparison with the nutritional balances reached in choice situations. Heatmaps on 3D nutritional geometric framework representing: (a,b) adult survival, at 30°C (upper panel) and 20°C (bottom panel). Warmer colours indicate better performances. Small coloured circles are the nutrient collection obtained in choice experiment for each micro-colony, while big coloured circles are their medians.

(c) 30°C Relative weight



(d) 20°C Relative weight

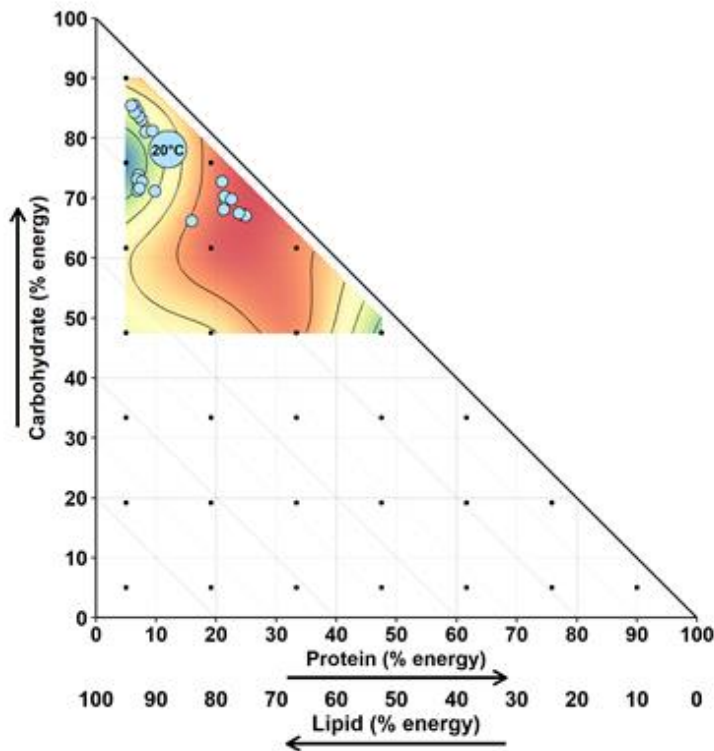
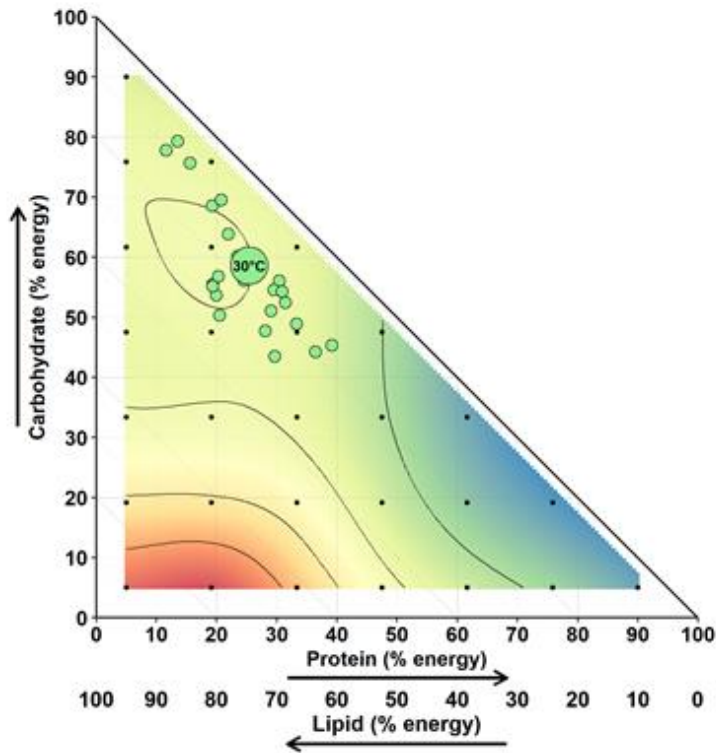


Figure 27: Performance traits in no-choice situations and comparison with the nutritional balances reached in choice situations. Heatmaps on 3D nutritional geometric framework representing: (c,d) the relative dry weight (in mg) for a median bee (of 4.5mm thorax width) and at 30°C (upper panel) and 20°C (bottom panel). Warmer colours indicate better performances. Small coloured circles are the nutrient collection obtained in choice experiment for each micro-colony, while big coloured circles are their medians.

(e) 30°C Egg-laying



(f) 20°C Egg-laying

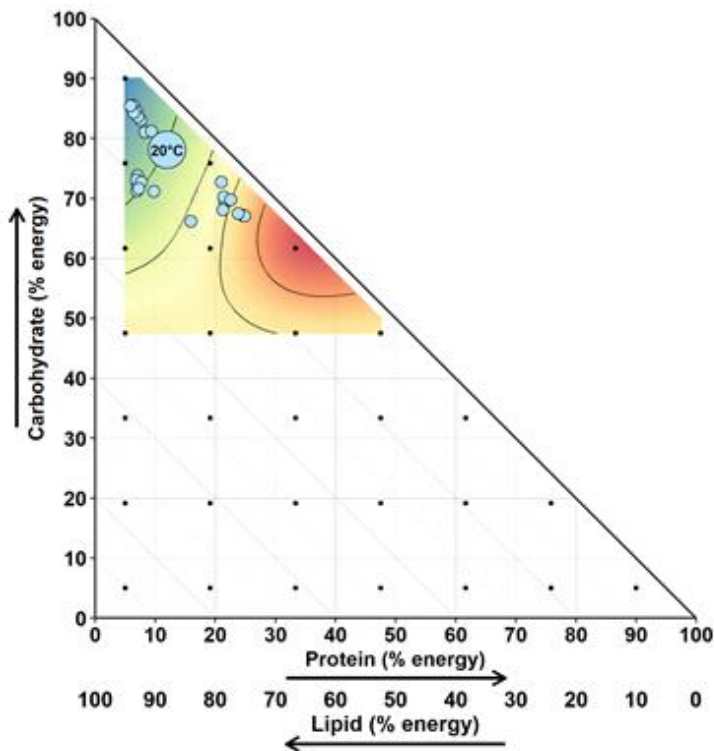


Figure 27: Performance traits in no-choice situations and comparison with the nutritional balances reached in choice situations. Heatmaps on 3D nutritional geometric framework representing: (e,f) laid eggs (total number of eggs and larvae) at 30°C (upper panel) and 20°C (bottom panel). Warmer colours indicate better performances. Small coloured circles are the nutrient collection obtained in choice experiment for each micro-colony, while big coloured circles are their medians.

Discussion

Previous studies showed bees regulate their intake of nutrients from multiple food sources in stable laboratory conditions (Stabler et al., 2015; Vaudo et al., 2016b; Kraus et al., 2019a). Here we studied how bumblebees regulate their collection of three macronutrients under various temperature regimes (20°C; 30°C; 35°C). We found that an elevation in temperature increased the total amount of food collected by bumblebees. Bumblebees adopted various nutritional behaviours as temperature changed: while at 30°C they achieved a precise nutritional intake of 25:59:16 P:C:L (% energy), they searched for the most carbohydrate-rich diets at 20°C, and instead collected more lipids and/or water at 35°C. Comparing choice and no-choice experiments clearly shows that bumblebees prioritize workers' weight at 30°C, while at 20°C they favoured survival.

Bumblebees balance between nutrients at 30°C to develop their reproductive ability

Our results demonstrate that bumblebees at 30°C preferred to achieve a precise nutrition balance between carbohydrates, proteins and lipids, rather than focusing on carbohydrates for example. Even though carbohydrates were predominantly collected (59%), proteins represented a quarter of collected diet. Since proteins and amino-acids are significant resources for egg-laying and larval growth (Tasei and Aupinel, 2008a; Moerman et al., 2016; 2017), this is consistent with the optimal temperature for brood development being around 30°C (Gürel and Gösterit, 2008; Nasir et al., 2019). Lipids, in particular sterol, also play an important role in colony development (Vanderplanck et al., 2014; Moerman et al., 2017), but they were collected in lesser amounts (16%), yet possibly sufficient in our conditions. It should be noted that these conclusions result from observation at the collective of micro-colonies, so that we cannot know if all individuals of a same micro-colony made the same nutritional choices, or if some specialized in collecting proteins (e.g., for egg-laying) while others focused on carbohydrates for their own survival. This is possible, because queen's pheromones inhibit workers reproduction only if in presences of her own offspring and if she could be seen by workers (Orlova and Amsalem, 2021). Otherwise, in micro-colonies one

worker usually becomes dominant to replace the absent queen and lay an egg-cell within 2 weeks, which is the duration of our experiments (Cnaani et al., 2002; 2007; Tasei and Aupinel, 2008b). In general, dominant individuals will have larger and mature ovaries and will allocate lipid reserves for protein synthesis (e.g., the yolk protein vitellogenin) into their oocytes (Tufail et al., 2014). Bumblebees tested at 30°C selected a nutritional balance that maximized their relative weight (as revealed by no-choice experiments), which was correlated with ovarian maturation (Treonore et al., 2019). This indicate that they selected the food best suited for their own physiological development. Moreover, juvenile hormone, which level is associated with ovary maturation, seems to increase metabolic rate and food consumption in bumblebees (Shpigler et al., 2021) explaining the increase in food collection.

At a lower temperature, bees increased their carbohydrate intake and gave priority to survival

When confronted to a lower temperature (20°C), bumblebees collected less food and only searched for the diet richest in carbohydrates within those available in each choice configuration. An increased collection of lipids would have been expected as a way to produce more wax in order to isolate their nest from cold (Tulloch, 1970; Heinrich, 1979). Still, this was not the case, which may be explained by the absence of brood to protect (Nagari et al., 2019). Rather, the observed preference for carbohydrates corresponds to the nutritional strategy most favourable for survival, as shown by no-choice experiments. In parallel, we observed low levels of activity among workers at this lower temperature, which is reminiscent with the strategies evolved by bumblebee queens to save energy during overwintering stage (Heinrich, 2004; Goulson, 2010) by reducing their metabolic rate and activity levels (Hahn and Denlinger, 2007; 2011). Thus, it seems that bumblebees managed to adapt their behaviour and nutritional collection to get through cold periods.

Water and/or lipids needs increased at higher temperature

In our conditions, an increase in temperature to 35°C led to an increased collection of lipids, both in proportion and quantity. However, as more lipidic diets were also more

diluted, it is difficult to discriminate if bees here searched for more lipids, more water, or both. On the one hand, water collection is not usual in bumblebees, but has been already observed in *B. terrestris* during warm conditions (Ferry and Corbet, 1996). This may be a way to fulfil a water deficit in the colony (Nicolson, 2009) when dry and hot weather conditions lower the volume of nectar available from flowers (Descamps et al., 2021b). As such conditions are becoming more frequent due to global warming, this behaviour may occur more and more often, thus deserving further studies (Woodard, 2017). On the other hand, the need for more lipids might be increased as some of them could be used to limit body desiccation by contributing to the impermeability of the cuticle (Benoit, 2010; Chown et al., 2011). Also, bumblebee queens accumulate lipids just before winter (Votavová et al., 2015), likely as a source of energy storage for egg laying and development (Canavoso et al., 2001; Ziegler and Van Antwerpen, 2006). Thus, lipids are important to insects, as they play multiple functions, but their role with temperature elevation remains unclear. Further studies are necessary to support or discard possible explanations; for example, testing those diets against water could provide some insights.

Heat stress and/or lipid toxicity cause detrimental effects on bees

We observed a high mortality in bumblebees constrained at 35°C. This can be explained because, at such high external temperature, their internal thoracic temperature can increase up to near 45°C, their lethal limit (Heinrich, 1979). In addition, their development may be impaired if, as for example in honeybees, heat stress during pupal stage impairs the development of wings, legs and proboscis, as well as brain maturation and immunocompetence (Groh et al., 2004; Medina et al., 2018; 2020). However, another possible source of mortality at 35°C might be the increased intake of lipids that we observed. Indeed, diets containing too many lipids shorten lifespan (honeybees: Haddad et al., 2007; Manning et al., 2007; bumblebees: Vaudo et al., 2016b; Ruedenauer et al., 2020). Also, an excess in fatty acids can cause intoxication by damaging cell membranes (Canavoso et al., 2001; Haddad et al., 2007). Therefore, worker bumblebees in the laboratory usually perceive and select food on the basis of its lipid content to avoid such toxic effects (Ruedenauer et al., 2020). Indeed, we observed that

bumblebees did not change their ratio of lipids over carbohydrates and proteins at 20 and 30°C, maintaining a lipid percent of 14.5%. Here, at 35°C bumblebees might have increased their consumption of lipids as a side-effect of collecting more diluted diets to get more water, thus possibly getting intoxicated. They may also have suffered from the combination of heat stress and an increase of total lipids as well as proteins, also known to be toxic in insects (Lee et al., 2008a; Dussutour and Simpson, 2012). Further survival analyses on our data could help us to confirm this hypothesis.

Conclusion

Social insects adapt to temperature variations over the year, through changes in the nest architecture and thermoregulation behaviour (Jones and Oldroyd, 2006). However, frequencies and durations of brutal temperature changes (e.g., heat waves) are constantly increasing nowadays (Meehl and Tebaldi, 2004; McKechnie and Wolf, 2010). This fragilizes floral resources available to bumblebees and other pollinators (Goulson et al., 2015; Descamps et al., 2021b), thus limiting their foraging behaviour and leading to unbalanced nutrient intake. Bumblebees are sensitive to heat stress, but manifest a certain level of resilience through behavioural plasticity (e.g., by directly collecting water: Ferry and Corbet, 1996) and/or natural selection (e.g., by developing traits favouring resistance to heat such as a small body size and short hairs: Maebe et al., 2021; Zhao et al., 2021). Yet, ultimately, climate change drives the widespread decline in bumblebees (Kerr et al., 2015; Soroye et al., 2020) and already constitutes a risk of extinction of some species (Martinet et al., 2015). As temperature affects how bees select their diet, it becomes crucial to understand the link between nutrition and temperature in pollinators' behavior and physiology (Clissold and Simpson, 2015). A considerable number of studies and initiatives have begun to plant bee-friendly resources in response to agricultural practices and climate changes (Goulson et al., 2015; Hall et al., 2017; 2020). Identifying changes in intake targets of bees due to temperature variations during seasons and across extreme climatic events is necessary to better design and refine these actions.

This one is not my art. It was made by Dinho Bento, an amazing artist that gave me a lot of inspiration. I had the chance to work with him for the project Street Art Thesis, and absolutely do not regret it. The gorgeous bee cannot deny it.



GENERAL DISCUSSION

Knowledge input from thesis results

During my PhD, I studied the nutritional behaviours of bumblebees and their fitness consequences in various environmental and social conditions. The aim was to determine whether pollination services could be improved via the nutritional manipulation of commercial colonies. I discuss the limits and perspectives of my results in this final chapter.

By conducting nutritional assays in various experimental conditions in the lab and in the field, I could confirm that bumblebees were more responsive to carbohydrates than lipids or proteins, and that overall, their food collection tended to converge towards a well-defined nutritional target, irrespective of the age and body size of individuals. Yet, I also showed that these nutritional decisions were influenced by extreme environmental conditions. Microcolonies deprived of brood did not regulate protein intake anymore and over-collected it. They adapted their diet to survive at sub-optimal temperatures, by focusing on specific macronutrients. At a lower temperature, bumblebees searched for the most sugar-rich diets available, while at a very high temperature they collected more water and/or lipids.

Convergent proteins:lipids ratio over various environmental contexts and experimental designs

When we look at the proteins:lipids ratio in our experiments where bumblebees had a nutritional choice, we found congruent results with three of the experiments of Vaudo and collaborators. These authors analysed the nutritional content of pollens freely collected in various environments ranging from forests to valleys (Vaudo et al., 2018; 2020), as well as of natural or nutritionally modified pollens made available under constrained conditions in a flight arena (Vaudo et al., 2016a). In most scenarios (including ours), bumblebees showed a preference for collecting nutrients at a ratio of about 4:1 P:L (Figure 28). Only in two exceptions did we observe diverging ratios: a 1:5 ratio in our own experiment on bees foraging on artificial flowers (Chapter 1.C) and a 14:1 and 12:1 ratio in an experiment from the latter study on *B. terrestris* and *B.*

impatiens microcolonies, respectively, fed with artificial diets (Vaudo et al. 2016b). However, it should be noted that both experiments were performed using artificial diets that were poor in both proteins and lipids compared to carbohydrates, at concentrations from which bees might not be able to regulate the two nutrients. The nutrient ratio selected by bumblebees should correspond to the one maximising fitness traits, as suggested in Chapter 3. We found bumblebees fed diet ratio of 4:1 P:L were heavier than others, meaning they developed themselves more than other bumblebees fed with sub-optimal diet. Further analysis is required to determine if this weight gain was for oocyte development leading to egg-laying, for lipid storage, or for both.

Vaudo et al. mentioned that the consistent P:L ratio found in their experiments could be due to an active choice from the foragers between various floral patches, or to an average floral lipid content obtained by random collection (Vaudo et al., 2018). They subsequently evaluated the content in proteins and lipids of pollens recovered from diverse flower species or from pollen loads collected by three bee species (*Apis mellifera*, *Bombus impatiens*, and *Osmia cornifrons*). They obtained median and mean values of 1.7:1 and 2.5:1, respectively (Vaudo et al., 2020). Thus, the ratio favoured by bumblebees is higher than those average values, suggesting an active choice. Our own results, provide a strong support for this hypothesis, indicating that a P:L ratio of 4:1 indeed result from a non-random choice based on available diets (Figure 28).

Coevolution for the increase of proteins in plant

Plants that depend on insect pollinators generally have a higher P:L ratio in their pollen than wind-pollinated plants (Ruedenauer et al., 2019b). For example, pistachio (*Pistacia vera*) that mostly relies on wind-pollination, have pollens with ratio of 1:2.1, compared to sunflower (*Helianthus annuus*) or pumpkins (*Cucurbita pepo*) whose pollens have ratios of 1:1 and 1.5:1 respectively. This trend is even amplified with buzz-pollinated crops that needs bumblebees and stinglessbees, such as kiwis (*Actinidia deliciosa*) with a ratio of 2.8:1 (Raw, 2000; Cooley and Vallejo-Marín, 2021), or peppers (*Capsicum annuum*) with ratios ranging between 3.5:1 and 6.5:1 depending on the varieties (Vaudo et al., 2020).

Nectars and pollens have to be rewarding enough to attract pollinators in plants that rely on their visit for pollination. This is notably accurate in plant species that do not provide nectar, like some buzz-pollinated plants, where pollen serves as the only reward for pollinators and has to be particularly rich in nutrients (Buchmann, 1983; Ruedenauer et al., 2019b). Most pollens analysed by Vaudo et al. had ratios inferior to 3:1, suggesting physiological, ecological and phylogenetic limits on pollen nutritional values, especially the addition of proteins (Ruedenauer et al., 2019b; Vaudo et al., 2020). This highlights the trade-off made by plant where pollens serve simultaneously as function for their reproduction and as pollinator reward (Roulston et al., 2000)

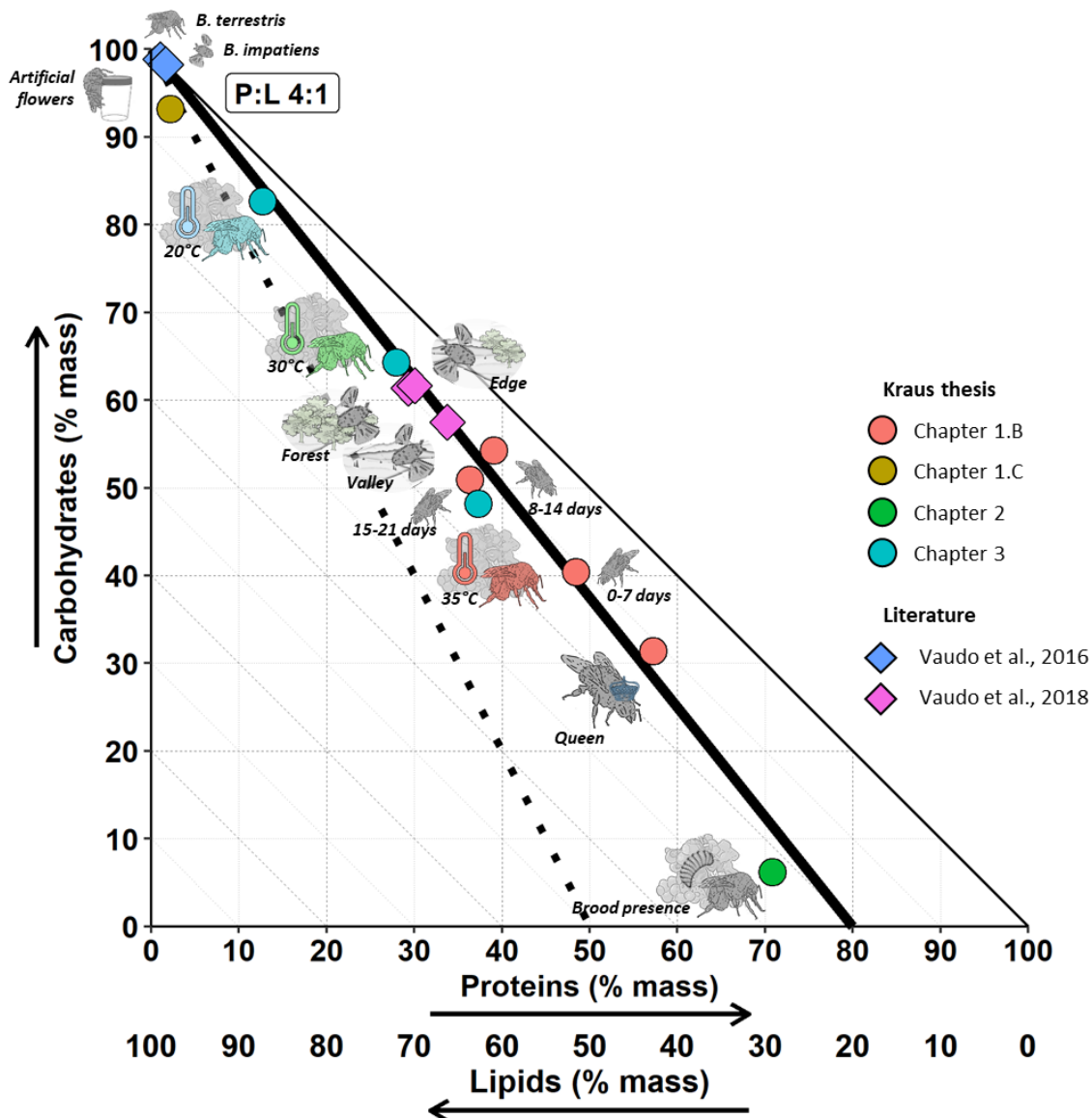


Figure 28: Congruent Proteins:Lipids ratios over experiments. Nutritional geometry model in three dimensions representing carbohydrates, proteins, and lipids collection by bumblebees. Coloured circles are the median of collected nutrient in various experiments from this thesis: from individuals of varying ages and castes in Chapter 1.B (red); foragers on artificial flowers in Chapter 1.C (yellow); micro-colonies with or without brood in Chapter 2 (green); to micro-colonies confronted to different temperatures in Chapter 3 (blue). Coloured diamonds show the results from the literature: of *B. terrestris* or *B. impatiens* tested on artificial diets (purple) (Vaudo et al., 2016b); and nutritional content of *B. impatiens* collected pollens either set in forest, valley, or the edge of the two (pink) (Vaudo et al., 2018). The black line corresponds to a ratio of 4:1 P:L, generally preferred by bumblebees (Vaudo et al., 2020). The dotted line corresponds to a ratio of 1:1 P:L.

Nutritional trade-off and behavioural adaptations

While controlling the proportion of lipids collected, we observed that bumblebees still adapted their collection in amount and balance of both carbohydrates and proteins to the experimental context. In fact, bumblebees displaced their nutritional state around the 4:1 P:L nutritional rails to adjust the proportion of carbohydrates to their needs (Figure 28). Under an optimal temperature for nest development (30°C), we observed a nutrient collection of 27:65:8 P:C:L (% mass) in Chapter 3. This collection had a diet ratio fitting the development of the reproductive function of heavier bees. Moreover, the observed collection was very similar to the ones analysed by Vaudo et al. in pollen grains collected by bumblebees set in forest, valley or in the edge (Vaudo et al., 2018). This confirms that bumblebees behaved with our artificial diets as if they had been bee-collected pollen with sugar addition, which foragers typically do to gather pollen grains into their corbiculae. On the other hand, bumblebees would move their collection toward carbohydrates when temperatures lower, giving up on egg-laying tasks to prioritize their survival.

In Chapter 1, regarding the nutritional choice experiment on individuals, we observed a similar switch toward more carbohydrates after the first week of emergence from the pupae, when the younger bees tended to have a similar diet as queens, rich in proteins and lipids, then started to collect more carbs after one week. Even though this result needs to be confirmed, it could be explained by the fact that nearly emerged bumblebees still need some proteinic input to fulfil their development (Skandalis et al., 2011; Jones et al., 2013), a similar age-adaption to the one observed in honeybees (Paoli et al., 2014a).

Finally, moving to higher extreme temperatures incited bumblebees to increase lipid collection. We speculate that the bees searched for water to resist the heat stress, forcing them to collect diets richer in lipids, which were also the most diluted. The need for water overrides the deterrent effect of lipids, leading to toxicity by over consumption. Providing water to bumblebees, as a decisive complementary diet, could help maintain colonies during heat waves and minimize their decline (Kerr et al., 2015; Martinet et al., 2015; Soroye et al., 2020).

From individual to colony nutrition

During this thesis, we principally measured food collection by bumblebees, but not ingestion. We had no possibility to discriminate when an individual ate what was collected, either directly from the feeders or in the honey pots in the nest. Thus, we could only overestimate their food ingestion, and to obtain a precise measure, we would have required the use of recent marking techniques (Řehoř et al., 2014; Macháčková et al., 2019).

A second point related to the one above is that collected food could be shared between individuals in the groups. Therefore, we could not know: first, if workers equally collected food over time, as it would require constant tracking of each individual; second, if there was an equal repartition of collected food in the groups, where one or two dominant workers could have eaten the majority of protein diets, and others had to be content with the remaining sugar diets, for example.

Moreover, colonies have a natural lifecycle where their social composition and nutritional needs vary over time, thus potentially modifying the workers' nutritional behaviour. As we observed in chapter 2, brood has a key influence on food collection by workers, since in its absence workers collected more proteins, suggesting that specific brood needs impose a specific regulation of protein intake. Besides, isolated individuals suffered from increased mortality as observed in Chapter 1.B, which highlights the importance of running experiments on the entire colony.

Automatic monitoring of colonies: a tool for research and agriculture

Moving from experiments on individuals or microcolonies to experiments on full colonies requires the collection of behavioural and fitness data from a large number of individuals over long periods of time. To face such a challenge in honeybees, researchers have developed experimental hives combined with bee tagging to continuously and automatically monitor the animals' behaviour in a natural context during day and night (Marchal et al., 2020). Such 'connected hives' aim to inform in real time about colony dynamics and environmental parameters. Indeed, they are typically equipped with: scales, the total colony weight being used as a proxy of foraging activity (Meikle et al.,

2008) or colony development (Flores et al., 2019); external and internal temperature and humidity sensors informing respectively on environmental conditions (which determine bees' activity: Seeley, 2014) and on the colony's capacity for thermoregulation; cameras recording bee traffic (Crall et al., 2018a; Klein et al., 2019) as well as nectar/pollen collection (Russell et al., 2017b) and social interactions (Wild et al., 2018) to investigate collective foraging strategies; and vibration/sound detection devices to explore in-hive communications (Rangel and Seeley, 2008). These sensors can be included in larger connected networks including connected objects in the environment such as artificial flowers and outdoor radar/cameras that precisely track spatial movement and foraging decisions of individuals (Lihoreau et al., 2012; Woodgate et al., 2017).

Adapting these methods to bumblebees opens the possibility of experimenting on entire colonies, as recently done to study the impact of neonicotinoids on bumblebees (Crall et al., 2018b). Such an approach was considered during the beginning of my thesis, but remained at the state of an attempt due to shipping delay and lockdowns from pandemic that reshaped my calendar and research priorities. Nevertheless, the work on connected colony prototype, where a bumblebee colony was set in boxes filled with sensors (Figure 29), helped us explore the various areas where it would be difficult to track by the sole eyes of the experimenter, as described above.

Tracking the nest size, number of larvae and workers via image processing (Figure 30) could address the question on how workers/larvae ratio will influence the nutritional needs of the colony and foragers choice. We hypothesized that during the initial first weeks of colony development, the nutritional needs of the colony would mostly meet those of the queens raising the first batch of workers. Then, as more workers emerge from pupae, the overall pollen and nectar collection should increase, but with a general bias toward carbohydrates to sustain the increasing nest activity. Finally, the "competition phase" of the colony would bias nutrient needs toward proteins as some workers would fight to develop their gonads and lay their own offspring.

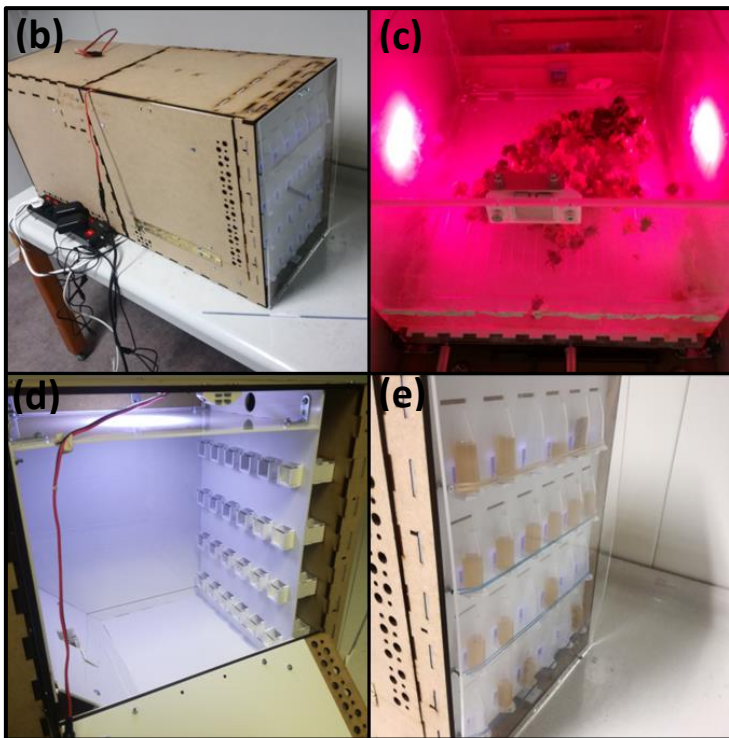
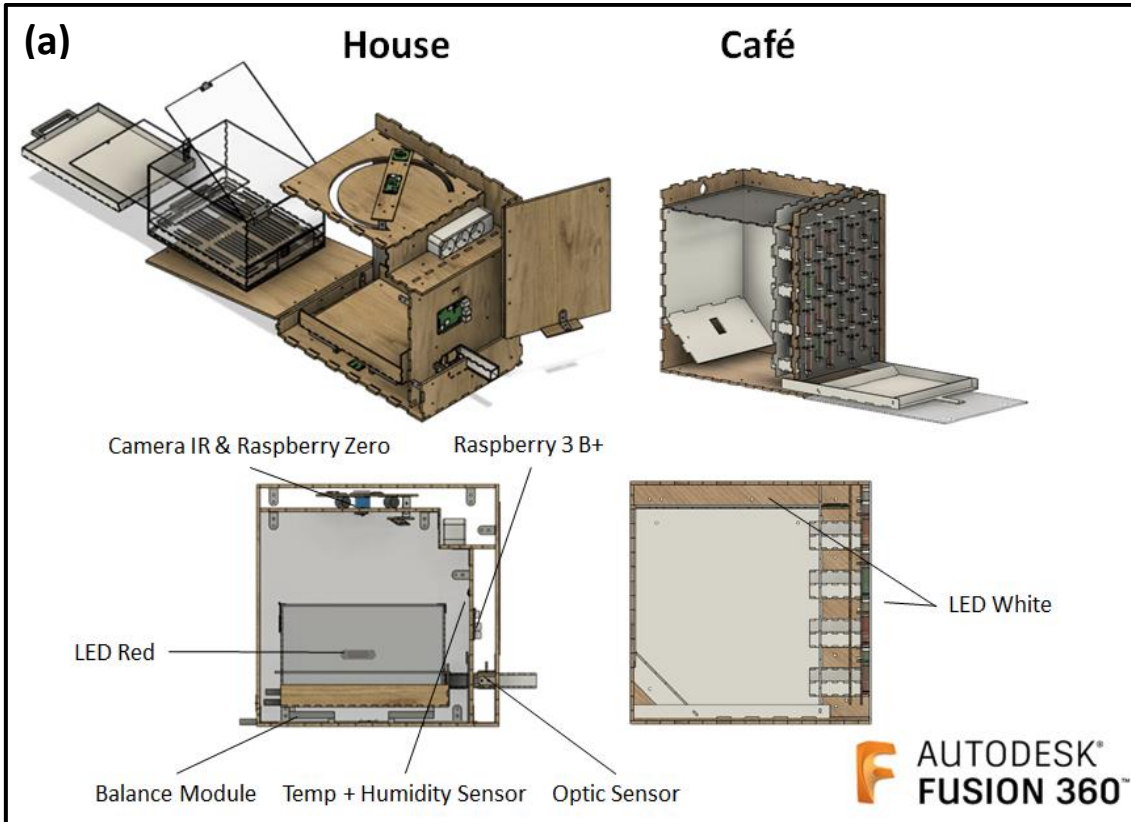


Figure 29: Bumblebee connected colony. (a) Schematic view and picture (b) of the connected colony. (c) Bumblebees were set in a nest area (transparent plastic box). It could be removed from a house area (wooden box), kept under red light and filled with sensors (four balances at each nest corner; two infra-red cameras; temperature and humidity sensors; two optic sensors for in/out activity). All sensors were monitored by two micro-computers (raspberry 3B+ and raspberry zero). (d) A house area was jointed with the café area (wooden box), kept under white light, and served as a foraging area. (e) Various food to bees (e.g., sugar solutions, syrups, pollens, solid powders) could be offered to bees in vials (drilled 5ml Eppendorf), set in the end of one of the 24 tunnels. Vials were blocked by a transparent wall that allowed us to track food collection.

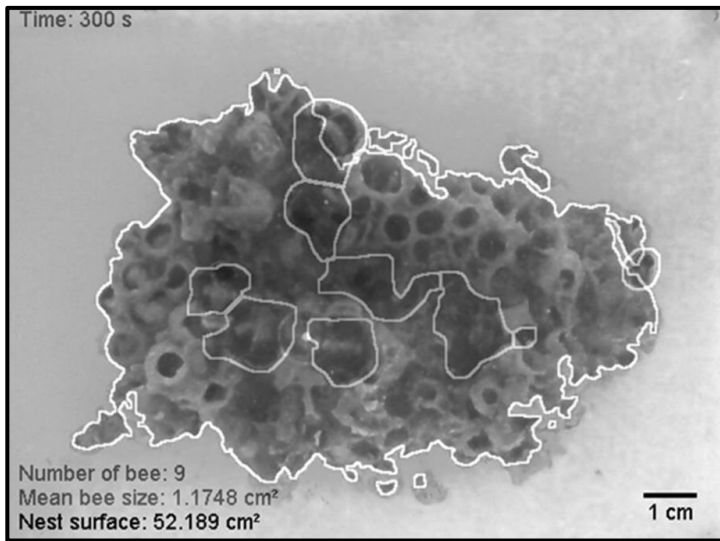
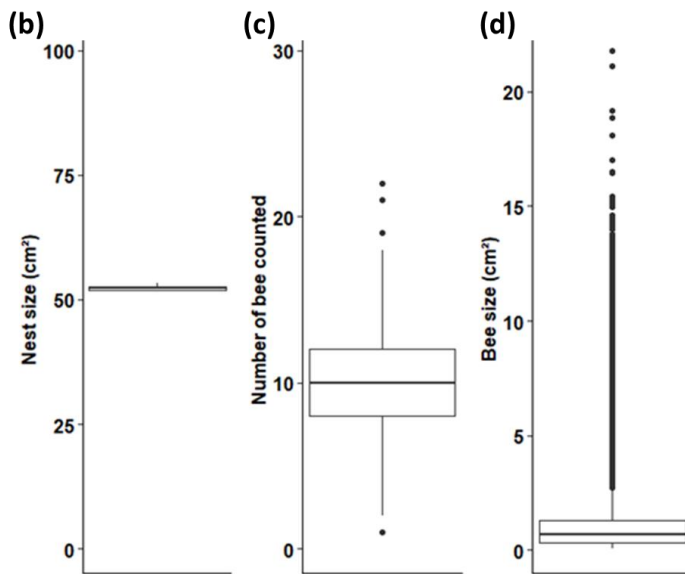


Figure 30: Pilot data: Tracking of bumblebee nest development. (a) Image a nest image taken from an in-hive camera, with automatic contouring of nest compartments using image analysis tools (Schneider et al., 2012). Estimates of (b) nest size (cm^2), (c) number of bees and (d) bees body size (cm^2) were obtained from 60 pictures, each taken every five seconds.



Continuous tracking of bees' in/out traffic would indicate the nutritional quality of food proposed in the foraging area for the entire colony. Coupling individual tagging and automatic recognition tools for each food source (e.g., detection of pollen loads from camera images) would give us insight on foraging repartition among workers. Knowing the foraging history of individual workers will allow us to confirm the supposed generalist behaviour for nutrient collection, and whether it varies across individuals and over time, as discussed in Chapter 1.

It becomes crucial to understand the behaviours and needs of these insects, considering the significant economic value of their pollination service in greenhouses, orchards and agricultural fields (Velthuis and van Doorn, 2006; Lautenbach et al., 2012). Monitored colonies appear to be a vital tool for research and agriculture, that put our study back into more natural and changing environment with entire colonies that evolved over time, and still guarantee a precise data collection. By helping to design strategies to maintain pollinator populations and to maximize pollination efficiency, nutritional ecology appears as a potent lever for actions.

Manipulating bee nutrition to improve crop pollination

My CIFRE thesis was co-funded by Koppert France (Cavaillon, France). This company develops and proposes bio-solutions, such as natural pest predators, micro-organisms, bio-stimulants and bumblebees to support, protect, strengthen and help the development and reproduction of food crops and ornamental plants. Pollination by bumblebees is a significant part of their activity, and Koppert continues to prospect on how to optimise their pollination service by producing colonies and deploying them in crops.

Over the past decades, research on bumblebee nutrition has brought considerable knowledge on how to breed bumblebee colonies (Velthuis and van Doorn, 2006) and what food to provide to them in order to maximise their development in laboratory conditions (Génissel et al., 2002; Tasei and Aupinel, 2008a; Moerman et al., 2016; 2017). But bumblebees face critical challenges in agricultural contexts, essentially caused by the reduced flower availability and diversity in time and space due to habitat fragmentation and monoculture practices (Howard et al., 2003; Goulson et al., 2015; St. Clair et al., 2020; Flowers et al., 2020). It thus becomes crucial to support them directly on the field to maintain an efficient pollination service. To do so, two possible approaches emerge from my work.

Boosting the number of foragers by using optimal diets

The first method is related to colony development. To mitigate nutritional stresses bees face in highly anthropized landscapes, researchers have proposed to increase floral resources (Goulson et al., 2008). Such strategies of nutritional complementation in agricultural field jointly increased local pollinator populations as well as crop pollination (Carvalho et al., 2012; Garibaldi et al., 2016). The advantage with commercial bumblebees is that we could feed them directly into their colony, and precisely control the proposed diet. Thus, as observed in the literature and in my experiments, providing them with an optimal diet that maximized bumblebees' workers production would greatly increase foragers numbers over time. This would guarantee a consequent colony lifespan in agricultural condition with continuous foragers workflow and an increased level of pollination.

A previous study compared the nutritional value and effects of pumpkin pollen (*Cucurbita pepo*) with two pollens commonly used in floral enhancement schemes in pumpkin agroecosystems, sunflowers (*Helianthus annuus*) and sunn hems (*Crotalaria juncea*) (Treanore et al., 2019). No clear difference was found regarding oocyte development when bumblebees were fed one of the three pollens. However, the authors observed a slight weight gain when the bumblebees were fed sunn hems and increased wax production when fed sunflowers. This emphasises the need to study other fitness traits in bees than just survival, such as egg-laying and larvae development to precisely assess the quality of pollens. Nevertheless, they observed that two floral resources used in enhancement schemes were low in proteins, with P:L ratios ranging from 1:1 to 1.7:1. We expect a pollen closer to a preferred P:L ratio of 4:1 by bumblebees, such as from poppy (*Papaver rhoeus*, 3.9:1) or white clover (*Trifolium repens*, 4.1:1) (Vaudo et al., 2020), would have a positive impact on weight gain, oocyte development and thus colony development, as demonstrated in chapter 3 (Figure 31). This example illustrates how we could take benefits from knowledge, such as the one arising from our experiments, on pollinators' nutritional preferences and constraints to improve the efficiency of these strategies.

Boosting the motivation of foragers using complementary diets

Nevertheless, feeding bumblebee colonies with an optimal diet could considerably reduce the motivation of foragers to visit real flowers. In fact, this loss of interest can already be observed for certain sub-optimal crops, such as pumpkins, where foragers would prefer to search for nutritional resources far away from the crops (Treanore et al., 2019). Therefore, rather than feeding colonies with an optimal diet, we should provide them with an unbalanced artificial diet which is nutritionally complementary to the pollen and nectar of the crop of interest. Thus, foragers will be inclined to concentrate their foraging activity on the targeted crops and avoid other flowers species in order to achieve an optimal diet for the colony. This method is based on the principle that bumblebees are able to balance their collection of several foods to achieve a nutritional target as observed in this thesis and previous studies (Stabler et al., 2015; Vaudo et al., 2016a,b; Kraus et al., 2019a).

If we come back to the previous example with pumpkin crop complementation, an efficient strategy would be to use pepper pollens that have a P:L ratio higher than the optimal one (6.5:1), as well as a sugar source, so that bumblebees could achieve their intake target by balancing their collection (Figure 31). Alternately, colonies could be directly fed with a nutritionally unbalanced diet but nutritionally complementary to pumpkin pollen to allow them to easily reach their intake target. In doing so, foragers should remain on the pumpkin crop rather than foraging on more distant food sources in search of complements outside the crop (Figure 31).

This is a perfect illustration of why it is decisive to precisely understand the nutritional needs of bee colonies at a given time, in order to propose a valuable complementary diet (Treanore et al., 2019). This is all the more important nowadays, as climate change disrupts flower availabilities and the quality of their resources (Høye et al., 2013; Miller-Struttman et al., 2015; Descamps et al., 2021b), which in turn influences the nutritional needs and behaviour of bumblebees (Chapter 3).

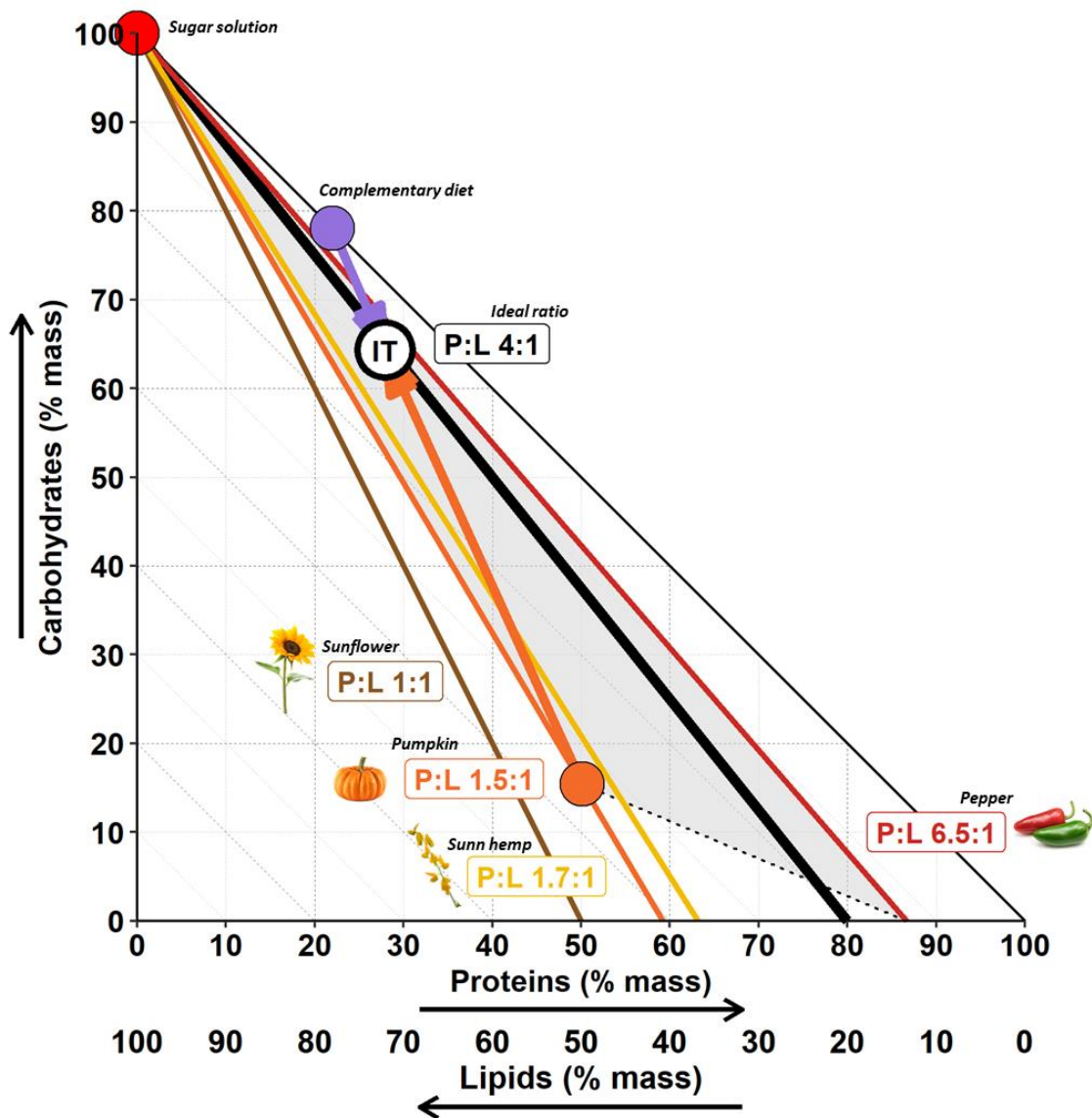


Figure 31: Possible application of my results for boosting crop pollination. Example of nutritional complementation for bumblebees foraging on pumpkins crops. Nutritional geometry model in three dimensions representing carbohydrates, proteins, and lipids collection by bumblebees. The black line corresponds to a ratio of 4:1 P:L, generally preferred by bumblebees (Vaudo et al., 2020). The white circle corresponds to the intake target (IT) of bumblebees measured in Chapter 3 at 30°C, optimal temperature for colony growth. The colour lines correspond to P:L ratio for pollens of sunflower (brown), pumpkin (orange), sunn hemp (yellow), and pepper (red) (Treanore et al., 2019). The orange circle corresponds to the nutrients present in pumpkins' pollen, the red circle to a sugar source, and the purple circle to a complementary diet of pumpkin pollens relative to the intake target of bumblebees (achieved via coloured arrows). The grey zone represents the nutritional environment sugar solution, pumpkin and pepper pollens create and that allow bumblebees to achieve their IT by selecting each of the three.

[Spike: This food tastes terrible as usual.

Jet: For being so bad you sure do eat a lot of it.

Spike: They say hunger is the best spice.]

-- Cowboy Bebop (1998)



References

Alford DV (1971) Egg laying by bumble bee queens at the beginning of colony development. *Bee World*, 52: 11–18.

Alford DV (1975) Bumblebees. *Davis-Poynter*.

Altaye SZ, Pirk CWW, Crewe RM, Nicolson SW (2010) Convergence of carbohydrate-biased intake targets in caged worker honeybees fed different protein sources. *Journal of Experimental Biology*, 213: 3311–3318.

Amdam G, Rueppell O (2006) Models of aging in honeybee workers. In: *Handbook of Models for Human Aging*, Elsevier Inc., 267–276.

Archer CR, Köhler A, Pirk CWW, Oosthuizen V, Apostolides Z, Nicolson SW (2014) Antioxidant supplementation can reduce the survival costs of excess amino acid intake in honeybees. *Journal of Insect Physiology*, 71: 78–86.

Arganda S, Bouchebti S, Bazazi S, Le Hesran S, Puga C, Latil G, Simpson SJ, Dussutour A (2017) Parsing the life-shortening effects of dietary protein: effects of individual amino acids. *Proceedings of the Royal Society B: Biological Sciences*, 284: 2016–2052.

Arien Y, Dag A, Zarchin S, Masci T, Shafir S (2015) Omega-3 deficiency impairs honey bee learning. *Proceedings of the National Academy of Sciences*, 112: 15761–15766.

Arien Y, Dag A, Shafir S (2018) Omega-6:3 Ratio More Than Absolute Lipid Level in Diet Affects Associative Learning in Honey Bees. *Front Psychol*, 9.

Ayasse M, Jarau S (2014) Chemical Ecology of Bumble Bees. *Annual Review of Entomology*, 59: 299–319.

Baker HG (1977) Non-Sugar Chemical Constituents of Nectar. *Apidologie*, 8: 349–356.

Baron GL, Jansen VAA, Brown MJF, Raine NE (2017) Pesticide reduces bumblebee colony initiation and increases probability of population extinction. *Nat Ecol Evol*, 1: 1308–1316.

Bazazi S, Arganda S, Moreau M, Jeanson R, Dussutour A (2016) Responses to nutritional challenges in ant colonies. *Animal behaviour*, 111: 235–249.

Behmer ST, Nes WD (2003) Insect sterol nutrition and physiology: a global overview. *Adv Insect Physiol*, 31: 1–72.

Behmer ST (2009) Insect herbivore nutrient regulation. *Annual review of entomology*, 54: 165–187.

Benoit JB (2010) Water Management by Dormant Insects: Comparisons Between Dehydration Resistance During Summer Aestivation and Winter Diapause. In:

Aestivation: Molecular and Physiological Aspects, (Arturo Navas C, Carvalho JE, Eds.). Springer, Berlin, Heidelberg 209–229.

Ben-Shahar Y, Robichon A, Sokolowski MB, Robinson GE (2002) Influence of gene action across different time scales on behavior. *Science*, 296: 741–744.

Bestea L, Réjaud A, Sandoz J-C, Carcaud J, Giurfa M, Brito Sanchez MG de (2021) Peripheral taste detection in honey bees: What do taste receptors respond to? *European Journal of Neuroscience*, 54: 4417–4444.

Boer SPA den., Duchateau MJHM (2006) A larval hunger signal in the bumblebee *Bombus terrestris*. *Insect Soc*, 53: 369–373.

Bradbury EJ, Wilkinson SJ, Cronin GM, Thomson PC, Bedford MR, Cowieson AJ (2014) Nutritional geometry of calcium and phosphorus nutrition in broiler chicks. Growth performance, skeletal health and intake arrays. *animal*, 8: 1071–1079.

Brito RM, McHale M, Oldroyd BP (2010) Expression of genes related to reproduction and pollen foraging in honey bees (*Apis mellifera*) narcotized with carbon dioxide. *Insect Molecular Biology*, 19: 451–461.

Brito Sanchez MG (2011) Taste perception in honey bees. *Chemical senses*, 36: 675–692.

Brodtschneider R, Crailsheim K (2010) Nutrition and health in honey bees. *Apidologie*, 41: 278–294.

Buchmann SL (1983) Buzz pollination in angiosperms. *Buzz pollination in angiosperms*, 73–113.

Burns JG, Dyer AG (2008) Diversity of speed-accuracy strategies benefits social insects. *Current biology*, 18: 953–954.

Burns JG., Foucaud J, Mery F (2011) Costs of memory: lessons from ‘mini’ brains. *Proceedings of the Royal Society B: Biological Sciences*, 278: 923–929.

Calderone NW, Robinson GE, Page RE (1989) Genetic structure and division of labor in honeybee societies. *Experientia*, 45: 765–767.

Canavoso LE, Jouni ZE, Karnas KJ, Pennington JE, Wells MA (2001) Fat Metabolism in Insects. *Annual Review of Nutrition*, 21: 23–46.

Cartar RV, Dill LM (1990) Colony energy requirements affect the foraging currency of bumble bees. *Behavioral Ecology and Sociobiology*, 27: 377–383.

Carvalho LG, Seymour CL, Nicolson SW, Veldtman R (2012) Creating patches of native flowers facilitates crop pollination in large agricultural fields: mango as a case study. *Journal of Applied Ecology*, 49: 1373–1383.

Chittka L, Thomson JD, Waser NM (1999) Flower constancy, insect psychology, and plant evolution. *Naturwissenschaften*, 86: 361–377.

- Chown SL, Sørensen JG, Terblanche JS (2011) Water loss in insects: An environmental change perspective. *Journal of Insect Physiology*, 57: 1070–1084.
- Clissold FJ, Simpson SJ (2015) Temperature, food quality and life history traits of herbivorous insects. *Current Opinion in Insect Science*, 11: 63–70.
- Cnaani J, Borst DW, Huang Z-Y, Robinson GE, Hefetz A (1997) Caste Determination in *Bombus terrestris*: Differences in Development and Rates of JH Biosynthesis between Queen and Worker Larvae. *Journal of Insect Physiology*, 43: 373–381.
- Cnaani J, Schmid-Hempel R, Schmidt JO (2002) Colony development, larval development and worker reproduction in *Bombus impatiens* Cresson. *Insectes soc*, 49: 164–170.
- Cnaani J, Wong A, Thomson JD (2007) Effect of group size on ovarian development in bumblebee workers (Hymenoptera: Apidae: *Bombus*). *Entomologia generalis*, 29: 305–314.
- Cohen AC (2015) *Insect Diets: Science and Technology*. CRC press.
- Colin T, Bruce J, Meikle WG, Barron AB (2018) The development of honey bee colonies assessed using a new semi-automated brood counting method: CombCount. *PLoS One*, 13: e0205816.
- Conradt L, Roper TJ (2005) Consensus decision making in animals. *Trends in Ecology & Evolution*, 20: 449–456.
- Coogan SC, Raubenheimer D (2016) Might macronutrient requirements influence grizzly bear–human conflict? Insights from nutritional geometry. *Ecosphere*, 7: e01204.
- Cook SC, Eubanks MD, Gold RE, Behmer ST (2010) Colony-level macronutrient regulation in ants: mechanisms, hoarding and associated costs. *Animal Behaviour*, 79: 429–437.
- Cooley H, Vallejo-Marín M (2021) Buzz-Pollinated Crops: A Global Review and Meta-analysis of the Effects of Supplemental Bee Pollination in Tomato. *Journal of Economic Entomology*, 114: 505–519.
- Couvillon MJ (2012) The dance legacy of Karl von Frisch. *Insect Soc*, 59: 297–306.
- Crailsheim K, Schneider LHW, Hrassnigg N, Bühlmann G, Brosch U, Gmeinbauer R, Schöffmann B (1992) Pollen consumption and utilization in worker honeybees (*Apis mellifera carnica*): dependence on individual age and function. *Journal of insect Physiology*, 38: 409–419.
- Crall JD, Gravish N, Mountcastle AM, Kocher SD, Oppenheimer RL, Pierce NE, Combes SA (2018a) Spatial fidelity of workers predicts collective response to disturbance in a social insect. *Nature Communications*, 9: 1201.

- Crall JD, Switzer CM, Oppenheimer RL, Versypt ANF, Dey B, Brown A, Eyster M, Guérin C, Pierce NE, Combes SA (2018b) Neonicotinoid exposure disrupts bumblebee nest behavior, social networks, and thermoregulation. *Science*, 362: 683–686.
- Cross WF, Hood JM, Benstead JP, Huryn AD, Nelson D (2015) Interactions between temperature and nutrients across levels of ecological organization. *Global Change Biology*, 21: 1025–1040.
- David Morgan E (2009) Trail pheromones of ants. *Physiological Entomology*, 34: 1–17.
- De Luca PA, Vallejo-Marín M (2013) What’s the ‘buzz’ about? The ecology and evolutionary significance of buzz-pollination. *Current Opinion in Plant Biology*, 16: 429–435.
- Descamps C, Quinet M, Baijot A, Jacquemart A-L (2018) Temperature and water stress affect plant–pollinator interactions in *Borago officinalis* (Boraginaceae). *Ecology and Evolution*, 8: 3443–3456.
- Descamps C, Jambrek A, Quinet M, Jacquemart A-L (2021a) Warm Temperatures Reduce Flower Attractiveness and Bumblebee Foraging. *Insects*, 12: 493.
- Descamps C, Quinet M, Jacquemart A-L (2021b) Climate Change–Induced Stress Reduce Quantity and Alter Composition of Nectar and Pollen From a Bee-Pollinated Species (*Borago officinalis*, Boraginaceae). *Frontiers in Plant Science*, 12.
- Detrain C, Deneubourg J-L (2008) Collective Decision-Making and Foraging Patterns in Ants and Honeybees. In: *Advances in Insect Physiology*, (Simpson SJ, Ed.). *Academic Press*, 123–173.
- Di Pasquale G, Salignon M, Le Conte Y, Belzunces LP, Decourtye A, Kretzschmar A, Suchail S, Brunet J-L, Alaux C (2013) Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? *PloS one*, 8: e72016.
- Dobson HEM (1988) Survey of Pollen and Pollenkitt Lipids—Chemical Cues to Flower Visitors? *American Journal of Botany*, 75: 170–182.
- Dornhaus A, Chittka L (2005) Bumble bees (*Bombus terrestris*) store both food and information in honeypots. *Behavioral Ecology*, 16: 661–666.
- Dreller C, Page Jr. RE, Fondrk MK (1999) Regulation of pollen foraging in honeybee colonies: effects of young brood, stored pollen, and empty space. *Behav Ecol Sociobiol*, 45: 227–233.
- Dubois T, Pasquaretta C, Barron AB, Gautrais J, Lihoreau M (2021) A model of resource partitioning between foraging bees based on learning. *PLoS computational biology*, 17: e1009260.
- Duchateau MJ, Velthuis HHW, Boomsma JJ (2004) Sex ratio variation in the bumblebee *Bombus terrestris*. *Behavioral Ecology*, 15: 71–82.

- Dussutour A, Simpson SJ (2008) Carbohydrate regulation in relation to colony growth in ants. *Journal of Experimental Biology*, 211: 2224–2232.
- Dussutour A, Simpson SJ (2009) Communal Nutrition in Ants. *Current Biology*, 19: 740–744.
- Dussutour A, Simpson SJ (2012) Ant workers die young and colonies collapse when fed a high-protein diet. *Proceedings of the Royal Society B: Biological Sciences*, 279: 2402–2408.
- Dussutour A, Poissonnier L-A, Buhl J, Simpson SJ (2016) Resistance to nutritional stress in ants: when being fat is advantageous. *Journal of Experimental Biology*, 219: 824–833.
- Elston C, Thompson HM, Walters KFA (2013) Sub-lethal effects of thiamethoxam, a neonicotinoid pesticide, and propiconazole, a DMI fungicide, on colony initiation in bumblebee (*Bombus terrestris*) micro-colonies. *Apidologie*, 44: 563–574.
- Feldhaar H (2014) Ant nutritional ecology: linking the nutritional niche plasticity on individual and colony-level to community ecology. *Current Opinion in Insect Science*, 5: 25–30.
- Ferry C, Corbet SA (1996) Water Collection by Bumble Bees. *Journal of Apicultural Research*, 35: 120–122.
- Fliszkiewicz M, Wilkaniec Z (2007) Fatty acids and amino acids in the fat body of bumblebee *Bombus terrestris* (L.) in diapausing and non-diapausing queens. *J Apic Sci*, 51: 55–63.
- Flores JM, Gil-Lebrero S, Gámiz V, Rodríguez MI, Ortiz MA, Quiles FJ (2019) Effect of the climate change on honey bee colonies in a temperate Mediterranean zone assessed through remote hive weight monitoring system in conjunction with exhaustive colonies assessment. *Science of The Total Environment*, 653: 1111–1119.
- Flowers B, Huang K-T, Aldana GO (2020) Analysis of the Habitat Fragmentation of Ecosystems in Belize Using Landscape Metrics. *Sustainability*, 12: 3024.
- Foster RL, Brunskill A, Verdirame D, O'Donnell S (2004) Reproductive physiology, dominance interactions, and division of labour among bumble bee workers. *Physiological Entomology*, 29: 327–334.
- Francis JS, Muth F, Papaj DR, Leonard AS (2016) Nutritional complexity and the structure of bee foraging bouts. *Behav Ecol*, 27: 903–911.
- Free JB (1970) The flower constancy of bumblebees. *The Journal of Animal Ecology*, 39: 395–402.
- Frisch K von (1967) The Dance Language and Orientation of Bees. *Harvard University Press*.

Gardner KE, Foster RL, O'Donnell S (2007) Experimental analysis of worker division of labor in bumblebee nest thermoregulation (*Bombus huntii*, Hymenoptera: Apidae). *Behavioral Ecology and Sociobiology*, 61: 783–792.

Garibaldi LA, Carvalheiro LG, Vaissière BE, Gemmill-Herren B, Hipólito J, Freitas BM, Ngo HT, Azzu N, Sáez A, Åström J, An J, Blochtein B, Buchori D, Garcia FJC, Oliveira da Silva F, Devkota K, Ribeiro MF, Freitas L, Gaglianone MC, Goss M, Irshad M, Kasina M, Filho AJSP, Kiill LHP, Kwapong P, Parra GN, Pires C, Pires V, Rawal RS, Rizali A, Saraiva AM, Veldtman R, Viana BF, Witter S, Zhang H (2016) Mutually beneficial pollinator diversity and crop yield outcomes in small and large farms. *Science*, 351: 388–391.

Gegeer RJ, Lavery TM (2005) Flower constancy in bumblebees: a test of the trait variability hypothesis. *Animal Behaviour*, 69: 939–949.

Génissel A, Aupinel P, Bressac C, Tasei J-N, Chevrier C (2002) Influence of pollen origin on performance of *Bombus terrestris* micro-colonies. *Entomologia Experimentalis et Applicata*, 104: 329–336.

Giraldeau L-A, Caraco T (2018) Social Foraging Theory. *Princeton University Press*.

Goulson D (2000) Are insects flower constant because they use search images to find flowers? *Oikos*, 88: 547–552.

Goulson D, Peat J, Stout JC, Tucker J, Darvill B, Derwent LC, Hughes WO (2002) Can alloethism in workers of the bumblebee, *Bombus terrestris*, be explained in terms of foraging efficiency? *Animal Behaviour*, 64: 123–130.

Goulson D, Lye GC, Darvill B (2008) Decline and Conservation of Bumble Bees. *Annual Review of Entomology*, 53: 191–208.

Goulson D (2010) Bumblebees: Behaviour, Ecology, and Conservation. *Oxford University Press* Oxford; New York.

Goulson D, Nicholls E, Botias C, Rotheray EL (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, 347: 1255957–1255957.

Goulson D, O'connor S, Park KJ (2018) The impacts of predators and parasites on wild bumblebee colonies. *Ecological Entomology*, 43: 168–181.

Goulson D (2019) The insect apocalypse, and why it matters. *Current Biology*, 29: 967–971.

Granero AM, Sanz JMG, Gonzalez FJE, Vidal JLM, Dornhaus A, Ghani J, Serrano AR, Chittka L (2005) Chemical compounds of the foraging recruitment pheromone in bumblebees. *Naturwissenschaften*, 92: 371–374.

Greenwald E, Segre E, Feinerman O (2015) Ant trophallactic networks: simultaneous measurement of interaction patterns and food dissemination. *Scientific Reports*, 5.

Groh C, Tautz J, Rössler W (2004) Synaptic organization in the adult honey bee brain is influenced by brood-temperature control during pupal development. *Proceedings of the National Academy of Sciences*, 101: 4268–4273.

Grüter C, Leadbeater E (2014) Insights from insects about adaptive social information use. *Trends in Ecology & Evolution*, 29: 177–184.

Gürel F, Gösterit A (2008) Effects of temperature treatments on the Bumblebee (*Bombus terrestris* L.) colony development. *Journal of the Faculty of Agriculture of Akdeniz University (Turkey)*.

Haddad LS, Kelbert L, Hulbert AJ (2007) Extended longevity of queen honey bees compared to workers is associated with peroxidation-resistant membranes. *Experimental Gerontology*, 42: 601–609.

Hagbery J, Nieh JC (2012) Individual lifetime pollen and nectar foraging preferences in bumble bees. *Naturwissenschaften*, 99: 821–832.

Hahn DA, Denlinger DL (2007) Meeting the energetic demands of insect diapause: Nutrient storage and utilization. *Journal of Insect Physiology*, 53: 760–773.

Hahn DA, Denlinger DL (2011) Energetics of insect diapause. *Annual review of entomology*, 56: 103–121.

Hall DM, Camilo GR, Tonietto RK, Ollerton J, Ahrné K, Arduser M, Ascher JS, Baldock KC, Fowler R, Frankie G (2017) The city as a refuge for insect pollinators. *Conservation Biology*, 31: 24–29.

Hall DM, Martins DJ (2020) Human dimensions of insect pollinator conservation. *Current Opinion in Insect Science*, 38: 107–114.

Heinrich B, Mudge PR, Deringis PG (1977) Laboratory analysis of flower constancy in foraging bumblebees: *Bombus ternarius* and *B. terrestris*. *Behavioral Ecology and Sociobiology*, 2: 247–265.

Heinrich B (1979) *Bumblebee Economics*. *Harvard University Press*.

Helm BR, Slater GP, Rajamohan A, Yocum GD, Greenlee KJ, Bowsher JH (2017) The geometric framework for nutrition reveals interactions between protein and carbohydrate during larval growth in honey bees. *Biology Open*, 6: 872–880.

Hendriksma HP, Shafir S (2016) Honey bee foragers balance colony nutritional deficiencies. *Behavioral Ecology and Sociobiology*, 70: 509–517.

Herbert EW, Svoboda JA, Thompson MJ, Shimanuki H (1980) Sterol utilization in honey bees fed a synthetic diet: Effects on brood rearing. *Journal of Insect Physiology*, 26: 287–289.

Hewson-Hughes AK, Hewson-Hughes VL, Miller AT, Hall SR, Simpson SJ, Raubenheimer D (2011) Geometric analysis of macronutrient selection in the adult domestic cat, *Felis catus*. *Journal of Experimental Biology*, 214: 1039–1051.

Hewson-Hughes AK, Hewson-Hughes VL, Colyer A, Miller AT, McGrane SJ, Hall SR, Butterwick RF, Simpson SJ, Raubenheimer D (2013) Geometric analysis of macronutrient selection in breeds of the domestic dog, *Canis lupus familiaris*. *Behavioral ecology*, 24: 293–304.

Hölldobler B, Wilson EO (2009) *The Superorganism: The Beauty, Elegance, and Strangeness of Insect Societies*. *WW Norton & Company*.

Hothorn T, Bretz F, Westfall P, Heiberger RM, Schuetzenmeister A, Scheibe S, Hothorn MT (2016) Package ‘multcomp.’ *Simultaneous inference in general parametric models Project for Statistical Computing, Vienna, Austria*.

Howard DC, Watkins JW, Clarke RT, Barnett CL, Stark GJ (2003) Estimating the extent and change in Broad Habitats in Great Britain. *Journal of Environmental Management*, 67: 219–227.

Høye TT, Post E, Schmidt NM, Trøjelsgaard K, Forchhammer MC (2013) Shorter flowering seasons and declining abundance of flower visitors in a warmer Arctic. *Nature Clim Change*, 3: 759–763.

Jandt JM, Dornhaus A (2009) Spatial organization and division of labour in the bumblebee *Bombus impatiens*. *Animal Behaviour*, 77: 641–651.

Jandt JM, Huang E, Dornhaus A (2009) Weak specialization of workers inside a bumble bee (*Bombus impatiens*) nest. *Behav Ecol Sociobiol*, 63: 1829–1836.

Jandt JM, Robins NS, Moore RE, Dornhaus A (2012) Individual bumblebees vary in response to disturbance: a test of the defensive reserve hypothesis. *Insect Soc*, 59: 313–321.

Jandt JM, Bengston S, Pinter-Wollman N, Pruitt JN, Raine NE, Dornhaus A, Sih A (2014) Behavioural syndromes and social insects: personality at multiple levels. *Biological Reviews*, 89: 48–67.

Jang T, Lee KP (2018) Comparing the impacts of macronutrients on life-history traits in larval and adult *Drosophila melanogaster*: the use of nutritional geometry and chemically defined diets. *Journal of Experimental Biology*, 221: jeb.181115.

Jeanson R, Weidenmüller A (2014) Interindividual variability in social insects—proximate causes and ultimate consequences. *Biological Reviews*, 89: 671–687.

Jones BM, Leonard AS, Papaj DR, Gronenberg W (2013) Plasticity of the worker bumblebee brain in relation to age and rearing environment. *Brain, behavior and evolution*, 82: 250–261.

- Jones JC, Oldroyd BP (2006) Nest Thermoregulation in Social Insects. In: Advances in Insect Physiology, (Simpson SJ, Ed.). *Academic Press*, 153–191.
- Kelemen E, Dornhaus A (2018) Lower temperatures decrease worker size variation but do not affect fine-grained thermoregulation in bumble bees. *Behavioral Ecology and Sociobiology*, 72: 1–8.
- Kerr JT, Pindar A, Galpern P, Packer L, Potts SG, Roberts SM, Rasmont P, Schweiger O, Colla SR, Richardson LL, Wagner DL, Gall LF, et al. (2015) Climate change impacts on bumblebees converge across continents. *Science*, 349: 177–180.
- Kim W, Gilet T, Bush JWM (2011) Optimal concentrations in nectar feeding. *PNAS*, 108: 16618–16621.
- Kim KE, Jang T, Lee KP (2020) Combined effects of temperature and macronutrient balance on life-history traits in *Drosophila melanogaster*: implications for life-history trade-offs and fundamental niche. *Oecologia*,.
- Kishani Farahani H, Moghadassi Y, Pierre J-S, Kraus S, Lihoreau M (2021) Poor adult nutrition impairs learning and memory in a parasitoid wasp. *Scientific reports*, 11: 1–11.
- Klein S, Cabirol A, Devaud J-M, Barron AB, Lihoreau M (2017) Why Bees Are So Vulnerable to Environmental Stressors. *Trends in Ecology & Evolution*, 32: 268–278.
- Klein S, Pasquaretta C, He XJ, Perry C, Søyvik E, Devaud J-M, Barron AB, Lihoreau M (2019) Honey bees increase their foraging performance and frequency of pollen trips through experience. *Sci Rep*, 9: 6778.
- Kraus S, Gómez-Moracho T, Pasquaretta C, Latil G, Dussutour A, Lihoreau M (2019a) Bumblebees adjust protein and lipid collection rules to the presence of brood. *Curr Zool*, 65: 437–446.
- Kraus S, Monchanin C, Gomez-Moracho T, Lihoreau M (2019b) Encyclopedia of Animal Cognition and Behavior.
- Lautenbach S, Seppelt R, Liebscher J, Dormann CF (2012) Spatial and Temporal Trends of Global Pollination Benefit. *PLOS ONE*, 7: e35954.
- Le Couteur DG, Solon-Biet S, Cogger VC, Mitchell SJ, Senior A, Cabo R, Raubenheimer D, Simpson SJ (2016) The impact of low-protein high-carbohydrate diets on aging and lifespan. *Cell Mol Life Sci*, 73: 1237–1252.
- Lee KP, Simpson SJ, Clissold FJ, Brooks R, Ballard JWO, Taylor PW, Soran N, Raubenheimer D (2008a) Lifespan and reproduction in *Drosophila*: New insights from nutritional geometry. *Proceedings of the National Academy of Sciences*, 105: 2498–2503.
- Lee KP, Simpson SJ, Wilson K (2008b) Dietary protein-quality influences melanization and immune function in an insect. *Functional Ecology*, 22: 1052–1061.

Lee KP, Jang T, Ravzanaadii N, Rho MS (2015) Macronutrient balance modulates the temperature-size rule in an ectotherm. *The American Naturalist*, 186: 212–222.

Lihoreau M, Chittka L, Raine NE (2011) Trade-off between travel distance and prioritization of high-reward sites in traplining bumblebees: Distance reward trade-off in bees. *Functional Ecology*, 25: 1284–1292.

Lihoreau M, Raine NE, Reynolds AM, Stelzer RJ, Lim KS, Smith AD, Osborne JL, Chittka L (2012) Radar tracking and motion-sensitive cameras on flowers reveal the development of pollinator multi-destination routes over large spatial scales. *PLoS biology*, 10: e1001392.

Lihoreau M, Buhl J, Charleston MA, Sword GA, Raubenheimer D, Simpson SJ (2014) Modelling nutrition across organizational levels: From individuals to superorganisms. *Journal of Insect Physiology*, 69: 2–11.

Lihoreau M, Buhl J, Charleston MA, Sword GA, Raubenheimer D, Simpson SJ (2015) Nutritional ecology beyond the individual: a conceptual framework for integrating nutrition and social interactions. *Ecology Letters*, 18: 273–286.

Lihoreau M, Poissonnier L-A, Isabel G, Dussutour A (2016) *Drosophila* females trade off good nutrition with high-quality oviposition sites when choosing foods. *Journal of Experimental Biology*, 219: 2514–2524.

Lihoreau M, Charleston MA, Senior AM, Clissold FJ, Raubenheimer D, Simpson SJ, Buhl J (2017) Collective foraging in spatially complex nutritional environments. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 372: 20160238.

Lihoreau M, Gómez-Moracho T, Pasquaretta C, Costa JT, Buhl J (2018) Social nutrition: an emerging field in insect science. *Current Opinion in Insect Science*, 28: 73–80.

Lihoreau M, Devaud J-M, Dore A, Dubois T, Kraus S, Lacombrade M, Monchanin C, Moran A, Gomez-Moracho T (*in rev*) Why bumblebees have become model species in apidology: a brief history and perspectives. *Apidologie*.

Lii GY, Garlich JD, Rock GC (1975) Protein and energy utilization by the insect, *argyrotaenia velutinana* (walker) fed diets containing graded levels of an amino acid mixture. *Comparative Biochemistry and Physiology Part A: Physiology*, 52: 615–618.

Livesey JS, Constable C, Rawlinson WG, Robotham AM, Wright C, Hampshire AE, Klark EG, Borrows WA, Horsell D, Cresswell JE (2019) The power and efficiency of brood incubation in queenless microcolonies of bumble bees (*Bombus terrestris* L.). *Ecological Entomology*, 44: 601–609.

Machovsky-Capuska GE, Priddel D, Leong PH, Jones P, Carlile N, Shannon L, Portelli D, McEwan A, Chaves AV, Raubenheimer D (2016) Coupling bio-logging with nutritional geometry to reveal novel insights into the foraging behaviour of a plunge-diving marine predator. *New Zealand Journal of Marine and Freshwater Research*, 50: 418–432.

Macháčková L, Votavová A, Mikát M, Matějková S, Řehoř I, Gillarová S, Straka J (2019) Measurement of protein and sugar consumed by bumblebee larvae under standard and food stress conditions using lanthanide complexes. *Insect Soc*, 66: 245–256.

Machovsky-Capuska GE, Andrades R, Santos RG (2020) Debris ingestion and nutritional niches in estuarine and reef green turtles. *Marine Pollution Bulletin*, 153: 110943.

Maebe K, Hart AF, Marshall L, Vandamme P, Vereecken NJ, Michez D, Smagghe G (2021) Bumblebee resilience to climate change, through plastic and adaptive responses. *Global Change Biology*, 27: 4223–4237.

Maklakov AA, Simpson SJ, Zajitschek F, Hall MD, Dessmann J, Clissold F, Raubenheimer D, Bonduriansky R, Brooks RC (2008) Sex-Specific Fitness Effects of Nutrient Intake on Reproduction and Lifespan. *Current Biology*, 18: 1062–1066.

Maleszka R (2018) Beyond Royalactin and a master inducer explanation of phenotypic plasticity in honey bees. *Communications biology*, 1: 1–7.

Manning R (2006) Fatty acid composition of pollen and the effect of two dominant fatty acids (Linoleic and oleic) in pollen and flour diets on longevity and nutritional composition of honey bees (*Apis mellifera*). *BSc (Hons) Murdoch University*.

Manning R, Rutkay A, Eaton L, Dell B (2007) Lipid-enhanced pollen and lipid-reduced flour diets and their effect on the longevity of honey bees (*Apis mellifera* L.). *Australian Journal of Entomology*, 46: 251–257.

Marchal P, Buatois A, Kraus S, Klein S, Gomez-Moracho T, Lihoreau M (2020) Automated monitoring of bee behaviour using connected hives: Towards a computational apidology. *Apidologie*, 51: 356–368.

Martinet B, Lecocq T, Smet J, Rasmont P (2015) A Protocol to Assess Insect Resistance to Heat Waves, Applied to Bumblebees (*Bombus Latreille*, 1802). *PLOS ONE*, 10: e0118591.

Martinez A, Farina WM (2008) Honeybees modify gustatory responsiveness after receiving nectar from foragers within the hive. *Behavioral Ecology and Sociobiology*, 62: 529–535.

McKechnie AE, Wolf BO (2010) Climate change increases the likelihood of catastrophic avian mortality events during extreme heat waves. *Biology Letters*, 6: 253–256.

McMenamin AJ, Daughenbaugh KF, Parekh F, Pizzorno MC, Flenniken ML (2018) Honey Bee and Bumble Bee Antiviral Defense. *Viruses*, 10: 395.

Medina RG, Paxton RJ, De Luna E, Fleites-Ayil FA, Medina Medina LA, Quezada-Euán JJG (2018) Developmental stability, age at onset of foraging and longevity of Africanized honey bees (*Apis mellifera* L.) under heat stress (Hymenoptera: Apidae). *Journal of Thermal Biology*, 74: 214–225.

- Medina RG, Paxton RJ, Hernández-Sotomayor SMT, Pech-Jiménez C, Medina-Medina LA, Quezada-Euán JJG (2020) Heat stress during development affects immunocompetence in workers, queens and drones of Africanized honey bees (*Apis mellifera* L.) (Hymenoptera: Apidae). *Journal of Thermal Biology*, 89: 102541.
- Meehl GA, Tebaldi C (2004) More intense, more frequent, and longer lasting heat waves in the 21st century. *Science*, 305: 994–997.
- Meikle WG, Rector BG, Mercadier G, Holst N (2008) Within-day variation in continuous hive weight data as a measure of honey bee colony activity. *Apidologie*, 39: 694–707.
- Mery F, Kawecki TJ (2004) An operating cost of learning in *Drosophila melanogaster*. *Animal Behaviour*, 68: 589–598.
- Mery Frederic, Kawecki Tadeusz J. (2003) A fitness cost of learning ability in *Drosophila melanogaster*. *Proceedings of the Royal Society of London Series B: Biological Sciences*, 270: 2465–2469.
- Miller-Struttman NE, Geib JC, Franklin JD, Kevan PG, Holdo RM, Ebert-May D, Lynn AM, Kettenbach JA, Hedrick E, Galen C (2015) Functional mismatch in a bumble bee pollination mutualism under climate change. *Science*, 349: 1541–1544.
- Mitchell BK, Itagaki H, Rivet M-P (1999) Peripheral and central structures involved in insect gustation. *Microscopy research and technique*, 47: 401–415.
- Miyamoto T, Amrein H (2017) Gluconeogenesis: An ancient biochemical pathway with a new twist. *Fly*, 11: 218–223.
- Moerman R, Vanderplanck M, Fournier D, Jacquemart A-L, Michez D (2017) Pollen nutrients better explain bumblebee colony development than pollen diversity. *Insect Conservation and Diversity*, 10: 171–179.
- Moerman R, Vanderplanck M, Roger N, Decleves S, Wathelet B, Rasmont P, Fournier D, Michez D (2016) Growth rate of bumblebee larvae is related to pollen amino acids. *Journal of economic entomology*, 109: 25–30.
- Molet M, Chittka L, Stelzer RJ, Streit S, Raine NE (2008) Colony nutritional status modulates worker responses to foraging recruitment pheromone in the bumblebee *Bombus terrestris*. *Behavioral Ecology and Sociobiology*, 62: 1919–1926.
- Mommaerts V, Wäckers F, Smagghe G (2013) Assessment of Gustatory Responses to Different Sugars in Harnessed and Free-Moving Bumblebee Workers (*Bombus terrestris*). *Chem Senses*, 38: 399–407.
- Morimoto J, Lihoreau M (2019) Quantifying nutritional trade-offs across multidimensional performance landscapes. *The American Naturalist*, 193: E168–E181.
- Muller H, Chittka L (2008) Animal personalities: the advantage of diversity. *Current Biology*, 18: 961–963.

- Muth F, Breslow PR, Masek P, Leonard AS (2018) A pollen fatty acid enhances learning and survival in bumblebees. *Behavioral Ecology*, 29: 1371–1379.
- Nagari M, Gera A, Jonsson S, Bloch G (2019) Bumble Bee Workers Give Up Sleep to Care for Offspring that Are Not Their Own. *Current Biology*, 29: 3488-3493.
- Nasir M, Mohsan A, Ahmad M, Saeed S, Aziz MA, Imran M, Sheikh UAA (2019) Effect of Different Temperatures on Colony Characteristics of *Bombus terrestris* (Hymenoptera: Apidae). *PJZ*, 51.
- Nicolson SW (2009) Water homeostasis in bees, with the emphasis on sociality. *Journal of Experimental Biology*, 212: 429–434.
- Nicolson SW (2011) Bee food: the chemistry and nutritional value of nectar, pollen and mixtures of the two. *African Zoology*, 46: 197–204.
- Nicolson SW, Thornburg RW (2007) Nectar chemistry. In: Nectaries and Nectar, (Nicolson SW, Nepi M, Pacini E, Eds.). *Springer Netherlands*, Dordrecht 215–264.
- Núñez JA, Giurfa M (1996) Motivation and regulation of honey bee foraging. *Bee World*, 77: 182–196.
- O'donnell S, Reichardt M, Foster R (2000) Individual and colony factors in bumble bee division of labor (*Bombus bifarius nearcticus* Handl; Hymenoptera, Apidae). *Insectes Sociaux*, 47: 164–170.
- Orlova M, Amsalem E (2021) Bumble bee queen pheromones are context-dependent. *Sci Rep*, 11: 16931.
- Page Jr RE, Erber J, Fondrk MK (1998) The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). *Journal of Comparative Physiology A*, 182: 489–500.
- Pandey P, Ramegowda V, Senthil-Kumar M (2015) Shared and unique responses of plants to multiple individual stresses and stress combinations: physiological and molecular mechanisms. *Frontiers in Plant Science*, 6.
- Paoli PP, Donley D, Stabler D, Saseendranath A, Nicolson SW, Simpson SJ, Wright GA (2014a) Nutritional balance of essential amino acids and carbohydrates of the adult worker honeybee depends on age. *Amino Acids*, 46: 1449–1458.
- Paoli PP, Wakeling LA, Wright GA, Ford D (2014b) The dietary proportion of essential amino acids and Sir2 influence lifespan in the honeybee. *AGE*, 36.
- Pereboom JJM (2000) The composition of larval food and the significance of exocrine secretions in the bumblebee *Bombus terrestris*. *Insectes soc*, 47: 11–20.
- Pereboom JJM, Velthuis HHW, Duchateau MJ (2003) The organisation of larval feeding in bumblebees (Hymenoptera, Apidae) and its significance to caste differentiation. *Insectes soc*, 50: 127–133.

Petanidou T, Van Laere A, N. Ellis W, Smets E (2006) What shapes amino acid and sugar composition in Mediterranean floral nectars? *Oikos*, 115: 155–169.

Piper MDW, Blanc E, Leitão-Gonçalves R, Yang M, He X, Linford NJ, Hodginott MP, Hopfen C, Soutoukis GA, Niemeyer C, Kerr F, Pletcher SD, et al. (2014) Erratum: Corrigendum: A holidic medium for *Drosophila melanogaster*. *Nature Methods*, 11: 971–971.

Pirk CWW, Boodhoo C, Human H, Nicolson SW (2010) The importance of protein type and protein to carbohydrate ratio for survival and ovarian activation of caged honeybees (*Apis mellifera scutellata*). *Apidologie*, 41: 62–72.

Plowright RC, Thomson JD, Lefkovitch LP, Plowright CMS (1993) An experimental study of the effect of colony resource level manipulation on foraging for pollen by worker bumble bees (Hymenoptera: Apidae). *Canadian journal of zoology*, 71: 1393–1396.

Poissonnier L-A, Arganda S, Simpson SJ, Dussutour A, Buhl J (2018) Nutrition in extreme food specialists: An illustration using termites. *Functional Ecology*, 32:2531–2541.

Pond WG, Church DB, Pond KR, Schoknecht PA (2004) Basic Animal Nutrition and Feeding. *John Wiley & Sons*.

Raine NE, Chittka L (2007) Pollen foraging: learning a complex motor skill by bumblebees (*Bombus terrestris*). *Naturwissenschaften*, 94: 459–464.

Rangel J, Seeley TD (2008) The signals initiating the mass exodus of a honeybee swarm from its nest. *Animal Behaviour*, 76: 1943–1952.

Raubenheimer D (2011) Toward a quantitative nutritional ecology: the right-angled mixture triangle. *Ecological Monographs*, 81: 407–427.

Raubenheimer D, Simpson SJ (1993) The geometry of compensatory feeding in the locust. *Animal Behaviour*, 45: 953–964.

Raubenheimer D, Simpson SJ (2018) Nutritional ecology and foraging theory. *Current Opinion in Insect Science*, 27: 38–45.

Raw A (2000) Foraging Behaviour of Wild Bees at Hot Pepper Flowers (*Capsicum annuum*) and its Possible Influence on Cross Pollination. *Annals of Botany*, 85: 487–492.

Řehoř I, Macháčková L, Bučánková A, Matějková S, Černá K, Straka J (2014) Measuring the sugar consumption of larvae in bumblebee micro-colonies: a promising new method for tracking food economics in bees. *Apidologie*, 45: 116–128.

Rho MS, Lee KP (2017) Temperature-driven plasticity in nutrient use and preference in an ectotherm. *Oecologia*, 185: 401–413.

Ribeiro MF (1994) Growth in bumble bee larvae: relation between development time, mass, and amount of pollen ingested. *Canadian Journal of Zoology*, 72: 1978–1985.

Riddell CE, Mallon EB (2006) Insect psychoneuroimmunology: Immune response reduces learning in protein starved bumblebees (*Bombus terrestris*). *Brain, Behavior, and Immunity*, 20: 135–138.

Robinson GE (1992) Regulation of division of labor in insect societies. *Annual review of entomology*, 37: 637–665.

Rotheray EL, Osborne JL, Goulson D (2017) Quantifying the food requirements and effects of food stress on bumble bee colony development. *Journal of Apicultural Research*, 56: 288–299.

Rothman JM, Raubenheimer D, Chapman CA (2011) Nutritional geometry: gorillas prioritize non-protein energy while consuming surplus protein. *Biology letters*, 7: 847–849.

Rothnie NE, Palmer MV, Burke DG, Sang JP, Hilliard EP, Salisbury PA, Evans DE, Knox RB, Williams EG (1987) Comparative analysis of fatty acids in pollen and seed of rapeseed. *Phytochemistry*, 26: 1895–1897.

Rottler A-M, Schulz S, Ayasse M (2013) Wax Lipids Signal Nest Identity in Bumblebee Colonies. *J Chem Ecol*, 39: 67–75.

Roulston TH, Cane JH (2000) Pollen nutritional content and digestibility for animals. *Plant Systematics and Evolution*, 222: 187–209.

Roulston TH, Cane JH, Buchmann SL (2000) What Governs Protein Content of Pollen: Pollinator Preferences, Pollen-Pistil Interactions, or Phylogeny? *Ecological Monographs*, 70: 617.

Ruedenauer FA, Spaethe J, Leonhardt SD (2016) Hungry for quality—individual bumblebees forage flexibly to collect high-quality pollen. *Behavioral ecology and sociobiology*, 70: 1209–1217.

Ruedenauer FA, Leonhardt SD, Lunau K, Spaethe J (2019a) Bumblebees are able to perceive amino acids via chemotactile antennal stimulation. *J Comp Physiol A*.

Ruedenauer FA, Spaethe J, Kooi CJ van der, Leonhardt SD (2019b) Pollinator or pedigree: which factors determine the evolution of pollen nutrients? *Oecologia*,.

Ruedenauer FA, Raubenheimer D, Kessner-Beierlein D, Grund-Mueller N, Noack L, Spaethe J, Leonhardt SD (2020) Best be(e) on low fat: linking nutrient perception, regulation and fitness. *Ecology Letters*, 23: 545–554.

Russell AL, Papaj DR (2016) Artificial pollen dispensing flowers and feeders for bee behaviour experiments.

Russell AL, Buchmann SL, Papaj DR (2017a) How a generalist bee achieves high efficiency of pollen collection on diverse floral resources. *Behavioral Ecology*, 28: 991–1003.

- Russell AL, Morrison SJ, Moschonas EH, Papaj DR (2017b) Patterns of pollen and nectar foraging specialization by bumblebees over multiple timescales using RFID. *Sci Rep*, 7: 42448.
- Ryder JT, Cherrill A, Thompson HM, Walters KF (2021) Lower pollen nutritional quality delays nest building and egg laying in *Bombus terrestris audax* micro-colonies leading to reduced biomass gain. *Apidologie*, 52: 1033–1047.
- Schmickl T, Crailsheim K (2002) How honeybees (*Apis mellifera* L.) change their broodcare behaviour in response to non-foraging conditions and poor pollen conditions. *Behavioral Ecology and Sociobiology*, 51: 415–425.
- Schmickl T, Crailsheim K (2004) Inner nest homeostasis in a changing environment with special emphasis on honey bee brood nursing and pollen supply. *Apidologie*, 35: 249–263.
- Schmidt KC, Hunt BG, Smith CR (2012) Queen, worker, and male yellowjacket wasps receive different nutrition during development. *Insect Soc*, 59: 289–295.
- Schmitz OJ, Rosenblatt AE, Smylie M (2016) Temperature dependence of predation stress and the nutritional ecology of a generalist herbivore. *Ecology*, 97: 3119–3130.
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*, 9: 671–675.
- Seeley TD (2014) Honeybee Ecology: A Study of Adaptation in Social Life. *Princeton University Press*.
- Shik JZ, Gomez EB, Kooij PW, Santos JC, Wcislo WT, Boomsma JJ (2016) Nutrition mediates the expression of cultivar–farmer conflict in a fungus-growing ant. *Proceedings of the National Academy of Sciences*, 113: 10121–10126.
- Shpigler HY, Magory Cohen T, Ben-Shimol E, Ben-Betzalel R, Levin E (2021) Juvenile hormone functions as a metabolic rate accelerator in bumble bees (*Bombus terrestris*). *Hormones and Behavior*, 136: 105073.
- Simcock NK, Wakeling LA, Ford D, Wright GA (2017) Effects of age and nutritional state on the expression of gustatory receptors in the honeybee (*Apis mellifera*). *PloS one*, 12: e0175158.
- Simpson SJ, Raubenheimer D (1993) A multi-level analysis of feeding behaviour: the geometry of nutritional decisions. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences*, 342: 381–402.
- Simpson SJ, Raubenheimer D (2012) The Nature of Nutrition: A Unifying Framework from Animal Adaptation to Human Obesity. *Princeton University Press* Princeton.
- Simpson SJ, Clissold FJ, Lihoreau M, Ponton F, Wilder SM, Raubenheimer D (2015) Recent advances in the integrative nutrition of arthropods. *Annual Review of Entomology*, 60: 293–311.

- Skandalis DA, Roy C, Darveau C-A (2011) Behavioural, morphological, and metabolic maturation of newly emerged adult workers of the bumblebee, *Bombus impatiens*. *Journal of Insect Physiology*, 57: 704–711.
- Smeets P, Duchateau MJ (2001) Feeding behaviour in the bumblebee *Bombus terrestris*. *Belg J Zool*, 131: 119–126.
- Solms J (1969) Taste of amino acids, peptides, and proteins. *Journal of Agricultural and Food Chemistry*, 17: 686–688.
- Solon-Biet SM, McMahon AC, Ballard JWO, Ruohonen K, Wu LE, Cogger VC, Warren A, Huang X, Pichaud N, Melvin RG, Gokarn R, Khalil M, Turner N, Cooney G, Sinclair DA, Raubenheimer D, Le Couteur DG, Simpson SJ (2014) The Ratio of Macronutrients, Not Caloric Intake, Dictates Cardiometabolic Health, Aging, and Longevity in Ad Libitum-Fed Mice. *Cell Metabolism*, 19: 418–430.
- Solon-Biet SM, Walters KA, Simanainen UK, McMahon AC, Ruohonen K, Ballard JWO, Raubenheimer D, Handelsman DJ, Couteur DGL, Simpson SJ (2015) Macronutrient balance, reproductive function, and lifespan in aging mice. *PNAS*, 112: 3481–3486.
- Somerville DC, Nicol HI (2006) Crude protein and amino acid composition of honey bee-collected pollen pellets from south-east Australia and a note on laboratory disparity. *Australian Journal of Experimental Agriculture*, 46: 141.
- Somerville DC, Rural Industries Research and Development Corporation, NSW Agriculture (2001) Nutritional Value of Bee Collected Pollens: A Report for the Rural Industries Research and Development Corporation. *Rural Industries Research and Development Corp.* Barton, ACT.
- Somme L, Vanderplanck M, Michez D, Lombaerde I, Moerman R, Wathelet B, Wattiez R, Lognay G, Jacquemart A-L (2015) Pollen and nectar quality drive the major and minor floral choices of bumble bees. *Apidologie*, 46: 92–106.
- Soroye P, Newbold T, Kerr J (2020) Climate change contributes to widespread declines among bumble bees across continents. *Science*, 367: 685–688.
- Spaethe J, Weidenmüller A (2002) Size variation and foraging rate in bumblebees (*Bombus terrestris*). *Insectes sociaux*, 49: 142–146.
- St. Clair AL, Zhang G, Dolezal AG, O’Neal ME, Toth AL (2020) Diversified Farming in a Monoculture Landscape: Effects on Honey Bee Health and Wild Bee Communities. *Environmental Entomology*, 49: 753–764.
- Stabentheiner A, Kovac H, Brodschneider R (2010) Honeybee colony thermoregulation—regulatory mechanisms and contribution of individuals in dependence on age, location and thermal stress. *PLoS one*, 5: e8967.

- Stabler D, Paoli PP, Nicolson SW, Wright GA (2015) Nutrient balancing of the adult worker bumblebee (*Bombus terrestris*) depends on the dietary source of essential amino acids. *Journal of Experimental Biology*, 218: 793–802.
- Stökl J, Steiger S (2017) Evolutionary origin of insect pheromones. *Current Opinion in Insect Science*, 24: 36–42.
- Sun hao-L, Abudisilimu N, Yi H, Li S, Liu T-X, Jing X (2022) Understanding nutritive need in *Harmonia axyridis* larvae: Insights from nutritional geometry. *Insect Science*.
- Szczêsna T (2006) Protein content and amino acid composition of bee-collected pollen from selected botanical origins. *Journal of Apicultural Science*, 50: 81–90.
- Tasei J-N, Aupinel P (2008a) Nutritive value of 15 single pollens and pollen mixes tested on larvae produced by bumblebee workers (*Bombus terrestris* , Hymenoptera: Apidae). *Apidologie*, 39: 397–409.
- Tasei J-N, Aupinel P (2008b) Validation of a Method Using Queenless *Bombus terrestris* Micro-Colonies for Testing the Nutritive Value of Commercial Pollen Mixes by Comparison with Queenright Colonies. *J Econ Entomol*, 101: 1737–1742.
- Team RC (2013) R: A language and environment for statistical computing.
- Therneau TM, Lumley T (2015) Package ‘survival.’ *R Top Doc*, 128.
- Tobback J, Mommaerts V, Vandersmissen HP, Smagghe G, Huybrechts R (2011) Age- and task-dependent foraging gene expression in the bumblebee *Bombus terrestris*. *Archives of insect biochemistry and physiology*, 76: 30–42.
- Toth AL, Robinson GE (2005) Worker nutrition and division of labour in honeybees. *Animal behaviour*, 69: 427–435.
- Treanore ED, Vaudo AD, Grozinger CM, Fleischer SJ (2019) Examining the nutritional value and effects of different floral resources in pumpkin agroecosystems on *Bombus impatiens* worker physiology. *Apidologie*, 50: 542–552.
- Tufail M, Nagaba Y, Elgendy AM, Takeda M (2014) Regulation of vitellogenin genes in insects. *Entomological Science*, 17: 269–282.
- Tulloch AP (1970) The composition of beeswax and other waxes secreted by insects. *Lipids*, 5: 247–258.
- Vallejo-Marín M (2019) Buzz pollination: studying bee vibrations on flowers. *New Phytologist*, 224: 1068–1074.
- Vanderplanck M, Moerman R, Rasmont P, Lognay G, Wathelet B, Wattiez R, Michez D (2014) How Does Pollen Chemistry Impact Development and Feeding Behaviour of Polylectic Bees? *PLoS ONE*, 9: e86209.

Vaudo AD, Tooker JF, Grozinger CM, Patch HM (2015) Bee nutrition and floral resource restoration. *Current Opinion in Insect Science*, 10: 133–141.

Vaudo AD, Patch HM, Mortensen DA, Tooker JF, Grozinger CM (2016a) Macronutrient ratios in pollen shape bumble bee (*Bombus impatiens*) foraging strategies and floral preferences. *Proceedings of the National Academy of Sciences*, 113: E4035–E4042.

Vaudo AD, Stabler D, Patch HM, Tooker JF, Grozinger CM, Wright GA (2016b) Bumble bees regulate their intake of essential protein and lipid pollen macronutrients. *The Journal of Experimental Biology*, 219: 3962–3970.

Vaudo AD, Farrell LM, Patch HM, Grozinger CM, Tooker JF (2018) Consistent pollen nutritional intake drives bumble bee (*Bombus impatiens*) colony growth and reproduction across different habitats. *Ecology and Evolution*, 8: 5765–5776.

Vaudo AD, Tooker JF, Patch HM, Biddinger DJ, Coccia M, Crone MK, Fiely M, Francis JS, Hines HM, Hodges M, Jackson SW, Michez D, et al. (2020) Pollen Protein: Lipid Macronutrient Ratios May Guide Broad Patterns of Bee Species Floral Preferences. *Insects*, 11: 132.

Vaughan DM, Calderone NW (2002) Assessment of pollen stores by foragers in colonies of the honey bee, *Apis mellifera* L. *Insectes soc*, 49: 23–27.

Velthuis HH (2002) The historical background of the domestication of the bumble-bee, *Bombus terrestris*, and its introduction in agriculture. *Pollinating Bees-The conservation link between agriculture and nature Ministry of Environment, Sao Paulo, Brasil*, 177: 184.

Velthuis HHW, Doorn A van (2006) A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie*, 37: 421–451.

Votavová A, Tomčala A, Kofroňová E, Kudzejová M, Šobotník J, Jiroš P, Komzáková O, Valterová I (2015) Seasonal Dynamics in the Chemistry and Structure of the Fat Bodies of Bumblebee Queens. *PLOS ONE*, 10: e0142261.

Wagner DL, Grames EM, Forister ML, Berenbaum MR, Stopak D (2021) Insect decline in the Anthropocene: Death by a thousand cuts. *Proceedings of the National Academy of Sciences*, 118: e2023989118.

Wali JA, Raubenheimer D, Senior AM, Le Couteur DG, Simpson SJ (2021) Cardio-metabolic consequences of dietary carbohydrates: reconciling contradictions using nutritional geometry. *Cardiovascular research*, 117: 386–401.

Walton A, Jandt JM, Dornhaus A (2019) Guard bees are more likely to act as undertakers: variation in corpse removal in the bumble bee *Bombus impatiens*. *Insect Soc*, 66: 533–541.

- Watrous KM, Duennes MA, Woodard SH (2019) Pollen Diet Composition Impacts Early Nesting Success in Queen Bumble Bees *Bombus impatiens* Cresson (Hymenoptera: Apidae). *Environ Entomol*, 48: 711–717.
- Weidenmüller A, Kleineidam C, Tautz J (2002) Collective control of nest climate parameters in bumblebee colonies. *Animal Behaviour*, 63: 1065–1071.
- Weidenmüller A (2004) The control of nest climate in bumblebee (*Bombus terrestris*) colonies: interindividual variability and self reinforcement in fanning response. *Behavioral Ecology*, 15: 120–128.
- Weiner CN, Hilpert A, Werner M, Linsenmair KE, Blüthgen N (2010) Pollen amino acids and flower specialisation in solitary bees. *Apidologie*, 41: 476–487.
- Wild B, Sixt L, Landgraf T (2018) Automatic localization and decoding of honeybee markers using deep convolutional neural networks. *arXiv:180204557*.
- Wills BD, Chong CD, Wilder SM, Eubanks MD, Holway DA, Suarez AV (2015) Effect of Carbohydrate Supplementation on Investment into Offspring Number, Size, and Condition in a Social Insect. *PLoS One*, 10.
- Woodard SH (2017) Bumble bee ecophysiology: integrating the changing environment and the organism. *Current Opinion in Insect Science*, 22: 101–108.
- Woodgate JL, Makinson JC, Lim KS, Reynolds AM, Chittka L (2017) Continuous Radar Tracking Illustrates the Development of Multi-destination Routes of Bumblebees. *Sci Rep*, 7: 17323.
- Wright GA, Nicolson SW, Shafir S (2018) Nutritional Physiology and Ecology of Honey Bees. *Annual Review of Entomology*, 63: 327–344.
- Wynants E, Lenaerts N, Wäckers F, Van Oystaeyen A (2021) Thermoregulation dynamics in commercially reared colonies of the bumblebee *Bombus terrestris*. *Physiological Entomology*, 46: 110–118.
- Yew JY, Chung H (2015) Insect pheromones: An overview of function, form, and discovery. *Progress in Lipid Research*, 59: 88–105.
- Zhao H, Li G, Guo D, Li H, Liu Q, Xu B, Guo X (2021) Response mechanisms to heat stress in bees. *Apidologie*, 52: 388–399.
- Ziegler R, Van Antwerpen R (2006) Lipid uptake by insect oocytes. *Insect Biochemistry and Molecular Biology*, 36: 264–272.

