Behavioural Regulation of Mineral Salt Intake in the Adult Worker Honey Bee, *Apis mellifera*

BY

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A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Insect Behaviour & Nutrition

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ABSTRACT

Honey bees are important insect pollinators, which social existence displays remarkable physiological and behavioural traits. These are tightly controlled by dietary cues. Detection, selection and ingestion of food entail the regulation of nutrient intake that leads to nutritional homeostasis. This study was motivated by the lack of information on mineral salt feeding preferences and regulation by adult honey bees. Here, in laboratory-based assays, I assessed the behavioural responses associated with feeding behaviour of adult worker honey bees to eight prevalent minerals in pollen (K, Na, Mg, Ca, Fe, Zn, Cu, Mn). In Chapter 3, using the classical Proboscis Extension Reflex approach and drinking assays, I tested the gustatory responses of forager bees to single minerals in either water or nectar-like solutions at four levels of concentration. I found that foragers (mixed-age) can detect individual salts/metals mineral salts with responses depending on mineral identity. Overall, bees found low mineral levels in water phagostimulatory. But when in sucrose solutions, only high Mg, Fe and Cu were rejected. In Chapter 4, using choice cohorts, I tested whether newly-emerged bees preferred a "salty" vs. "unsalty" diet and assessed the effects of single minerals on consumption responses and survival over 6 days. I verified that young bees 1) perceived and selected specific minerals in food; 2) showed behavioural regulation of mineral intake, but not all minerals were regulated to the same extent; 3) not all minerals acted as phagostimulants at low levels, but were deterrent at sufficiently high levels. This work is one of the firsts to evaluate gustatory responses of minerals, especially metals, and, to my knowledge, the first to assess the dietary self-selection of salts and metal nutrients in the context of behavioural regulation of intake in adult worker honey bees. The current study lays the groundwork for exploring mineral salt requirements, feeding preferences and regulatory mechanisms of salt intake in honey bees. ***Keywords:*** *Apis mellifera, workers, taste model, Bertrand's rule, micronutrients, behavioural regulation, self-selection, gustation.*

RESUMO[1]

As abelhas são insectos polinizadores importantes cuja organização social apresenta aspectos fisiológicos e comportamentais notáveis. Estes aspectos, por sua vez, são rigorosamente controlados por estímulos alimentares externos. A regulação do consumo de nutrientes é efectuado através de mecanismos de detecção, selecção e ingestão que asseguram a homeostasia nutricional. Um dos grandes motivos para a realização deste estudo incide sobre a falta de informação existente relacionada com a ingestão de nutrientes minerais e de que forma estes influenciam a comportamento alimentar de abelhas adultas. Desta forma e em contexto laboratorial, decidi avaliar as respostas comportamentais de abelhas adultas associadas à ingestão individual dos oito minerais mais prevalentes no pólen (fonte principal de nutrientes na dieta das abelhas): potássio (K), sódio (Na), cálcio (Ca), magnésio (Mg), ferro (Fe), zinco (Zn), cobre (Cu) e manganês (Mn).

No Capítulo 3, através da abordagem clássica do "Reflexo da Extensão da Probóscide" e da aceitação/rejeição de ingestão, foi possível testar as respostas gustativas das abelhas campeiras através do estímulo de um único mineral diluído em diferentes concentrações quer em água ou numa solução açucarada (34% sacarose). Com este tipo de testes verificou-se que as abelhas campeiras adultas (com idades mistas, > 21 dias) detectaram minerais em solução. Estas respostas dependeram do tipo de mineral testado. Em suma, baixos níveis de minerais em água são fagoestimulantes (atraentes ao paladar) para as abelhas. Contudo, quando os mesmos estão presentes em soluções de açucaradas as abelhas detectaram apenas os minerais Mg, Fe e Cu, e quando presentes em elevadas concentrações, acabando por rejeitar o estímulo.

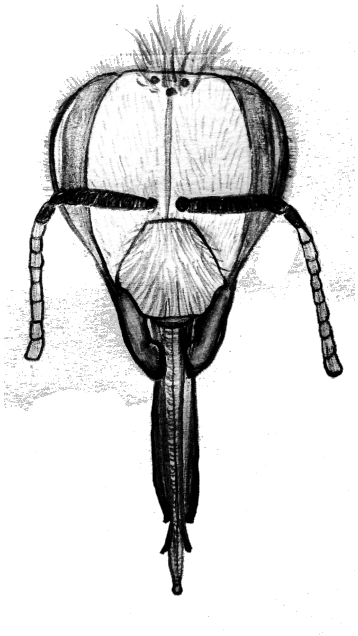
[1] Este texto não foi escrito em concordância com o acordo ortográfico Português.

No Capítulo 4, através da utilização de testes de consumo/alimentares em grupo, foi possível estudar a preferência e o comportamento alimentar que abelhas emergentes demonstram face a dietas "com sal" vs. dietas "sem sal". Simultaneamente, foram também avaliados os efeitos da ingestão de cada dieta mineral na sobrevivência das abelhas durante o ensaio (6 dias). Assim, observou-se que abelhas jovens (responsáveis por alimentar as larvas e a abelha rainha em contexto natural): 1) conseguiram detectar e escolher minerais específicos em dieta líquida; 2) demonstraram processos de regulação comportamental relativamente à ingestão de minerais, cujo grau de regulação dependeu do tipo de mineral 3) nem todos os minerais demonstraram ser fagoestimulantes quando presentes em baixas concentrações. Pelo contrário, determinados minerais dissuadiram a ingestão quando presentes em altas concentrações. Este é dos primeiro trabalhos que abordam a resposta gustativa de minerais em solução, em particular de metais, e, do meu conhecimento, o primeiro a avaliar a palatabilidade e escolha alimentar de nutrientes minerais em contexto de regulação de ingestão alimentar em abelhas obreiras adultas. O presente trabalho estabelece as bases para explorar em mais detalhe os requisitos nutricionais em sais minerais, preferências e mecanismos de regulação da ingestão de micronutrientes na abelha melífera.

***Palavras-chave**: Apis mellifera, micronutrientes, regulação comportamental, autoselecção, palatabilidade, insectos sociais, dieta, nutrição, abelha obreira, sais minerais, ingestão de alimentos.*

This thesis is dedicated to the honey bees,
who bestowed their lives on behalf of Science
by helping to understand them a little further.

To my grandfather "vermelhinho".



"For to the bee a flower is a fountain of life,
And to the flower a bee is a messenger of love,
And to both, bee and flower, the giving and the receiving of pleasure
is a need and an ecstasy."

by Kahlil Gibran, On Pleasure

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Now that this thesis is finally written, *Why aye man! Let's gannin the toon the neet ta git mortal!* A gigantic Portuguese hug to dearest and most fab Geordies in the Toon, Malcom and Terry! Thank you for being kind to me every day and since day 1. Thank you for making me feel at home and appreciated in this city. It surely has been a pleasure to get to know you. Not surprisingly, you were one of the reasons I decided to stay in Newcastle in first place. New adventures await us. Stay tuned!

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journey we withstood together and balanced each other. Thank you for listening, for cheering me up and for caring. Thank you for reading through my first raw chapter and the last one to be finished, and for your insightful comments.

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DECLARATION

I, postgraduate student no. S14006283, hereby declare that this thesis is grounded on original ideas and work designed and produced by myself, under the supervision and guidance of Professor Geraldine Wright. On specific occasions, undergraduate students Robyn Darnell and Victoria Blackham, and Mushtaq Al-Esawy (research technician) contributed to material preparation and/or executed subsets of experiments. Reference to ideas and work of others has been specifically acknowledged. Artwork throughout the thesis was produced by myself otherwise respective credits were acknowledged. This work has not contributed to any other degree.

Raquel Teixeira de Sousa

PREFACE

The starting point that lead to this point sparked around six years ago. I have always been driven to study biological phenomena, and observe our surrounding environments. Rui, my long-term partner and fellow biologist, was helping an uncle in keeping bees. Soon after, Rui's enthusiasm in keeping his own bees flourished, which led him to buy his first honey bee colonies in Northern Portugal.

Every weekend I would follow him to the hives, first to record those events through the lens and then to provide helpful assistance on managing and feeding the colonies. This interest kept growing, as well as the need to learn more about honey bee biology and the hard work processes involved in keeping bees. We attended several workshops on beekeeping practices (e.g. queen rearing, honey harvesting, supplemental feeding, honey bee health and pathologies), which in turn developed passion for the subject matter.

I was previously a research assistant at Universidade do Porto working on urban pollen allergenicity. During my usual week, I would walk around the city collecting flowers to analyse extracted pollen, then during the weekend, I would be an amateur photograph and assist Rui's beekeeping.

Through spending several hours observing honey bees working, foraging, grooming, stinging and feeding, I soon reached one conclusion. I would aim for a PhD as part of my career progression, and would study something relating to honey bees. It may sound rather romantic that even after learning of my subsequent allergy to honey bee venom, however, it felt (and still feels) like the right path to take. I not only enjoyed dealing with bees, but also learnt a number of facts about them by this time, which proved an advantage in beginning my future research.

One might questions "why the interest in the study of insects?". Insects (Family: *Insecta Linnaeus* 1758) are abundant throughout the world, variable in diversity and include six known orders; *Coleoptera* (e.g. beetles), *Diptera* (e.g. flies and mosquitoes), *Hemiptera* (e.g. true bugs), *Blattodea* (e.g. cockroaches and termites),

Lepidoptera (e.g. butterflies and moths) and *Hymenoptera* (e.g. wasps, ants and bees). Insects are critical to nutrient recycling in ecosystems, plant propagation, maintenance of food webs (for review (Gullan and Cranston, 2010), and as models for scientific research (e.g. fruit fly, *Drosophila* sp.). Additionally, insects are closely related to human economy, health, nutrition and culture. Some act as vectors of human diseases, whilst others directly contribute to our food production system and food security (Potts *et al.*, 2016). Insects have relatively short generation turnover, high fecundity, easy in-lab rearing and manipulation, and therefore are useful model organisms for studying general biological processes in their own right or to infer human biology. Conclusive evidence for suffering and pain in invertebrates is still absent. Though, humane care is always encouraged, insects (and other invertebrate animals) receive minimal ethical concerns still. Insects are, therefore, often preferred over vertebrate animals as models for several scientific experiments; for a review (Proctor, Carder and Cornish, 2013; Doke and Dhawale, 2015).

Honey bees are an especially good model animal and demonstrate an exquisite lifestyle, as a well-organized social insect society, and a pillar for our food production system. With this in mind my next step was securing a PhD scholarship and being accepted into a flexible program that wouldn't restrict studies on a previously defined line of research. Again, all this may sound rather presumptuous, but I was prepared for a challenge. Fortunately, a postgraduate program as such does exist, this being - The GABBA Program. After receiving my acceptance letter I was granted 4-year PhD scholarship funded by the Portuguese government and the European Union (FCT).

The GABBA Program is distinct in the way that it financially supports students to travel and visit research laboratories, targeted by students to conduct their doctoral research studies. In other words, it provides students with the less likely opportunity to visit and choose their "dream lab" before any formal commitment. Whilst "dream lab" seems far-fetched, it is only reasonable to admit that it is a privilege to have the opportunity to choose between teams that will work in accordance with your own personal and professional goals.

In late 2013, after a round trip visiting bee research labs in the USA, UK, Australia and Germany, I decided to take my research aims to Newcastle University, UK. There I joined Prof. Geraldine Wright's Lab, who kindly accepted me as part of her team and provided all the further support I required.

In 2014, I officially started my PhD project on adult honey bee nutrition and physiology. I have been fully committed until this very moment. Expectably, I was quite eager to start my research project by studying it all (behaviour, physiology, biochemistry and molecular studies) in three years! Eventually, I recollected senses and narrowed down my approach to something more realistic. I then, focused on testing how adult honey bees perceive mineral salts in food and how it translates into the duality of preference-aversion feeding thresholds that shape regulation of food intake. I conducted *ad nauseum* feeding assays using adult worker honey bees, testing their consumption and survival when given nectar-like diets laced with varying concentrations of single major salts (Na, K, Ca, Mg) or metals (Fe, Zn, Cu, Mn) present in bee natural food (pollen).

For the future, I wish to follow up this research as an academic and tackle other relevant questions of this subject matter. Furthermore, I anticipate these findings can also be integrated into the wealth of knowledge produced in Wright's Lab and improve the realm of bee nutrition products available in the market.

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LIST OF ABBREVIATIONS

Al	Aluminium
AmGr	*Apis mellifera*, Gustatory receptor
Ca	Calcium
Cd	Cadmium
Cl	Chloride
CS/US	Conditioned/Unconditioned Stimulus
Cu	Copper
ENaC	Epithelial Na Channels
ES	Ecological Stoichiometry
FAC	Ferric Ammonium Citrate
Fe	Iron
GEE	Generalized Estimating Equations
GMF	Geometric Framework for Nutrition
GRN	Gustatory Receptor Neuron
GRs	G-coupled gustatory receptor
GzLM	Generalized Linear Models
HPG	Hypopharyngeal Glands
HR	Hazard Ratio
IRs/IR76b	Ionotropic Receptor Channels
JH/Vg	Juvenile Hormone/Vitellogenin
K	Potassium
KM	Kaplan-Meier
LC50	Median Lethal Concentration, 50%
Li	Lithium
M	Molar (concentration)
Mg	Magnesium
mM	mili Molar (concentration)
Mn	Manganese
Na	Sodium
Ni	Nickel
P	Phosphorous
Pb	Lead
PCA	Principal Component Analysis

PER	(antennal) Proboscis Extension Reflex
ppk11/ppk19	pickpocket gene (low NaCl sensing)
ppm	Parts per million (concentration)
RJ	Royal Jelly
S	Sulfur
sano	*sano serrano* gene (high NaCl sensing)
Se	Selenium
SER	Sting Extension Reflex
SOD	Superoxide Dismutase
SWOT	Strengths, Weaknesses, Opportunities, Threats
Zn	Zinc
% w/v	Percent weight by volume (concentration)

1

General Introduction and Goals

Chapter 1

General Introduction

1.1 Abstract

Animal nutrition, specifically insect nutrition and regulatory strategies that lead to nutritional homeostasis, are topics of extensive research works and reviews (Dadd, 1973; Browne, 1975; Dethier, 1976; Newland, Cobb and Marion-Poll, 2009; Simpson and Raubenheimer, 2011; Lowe *et al.*, 2013). Most are focused on macronutrient regulation in several species, including social insects. Yet, very few have been dedicated to the detailed study of micronutrients such as mineral salts. In this Chapter my first goal is to drive the reader to revisit previous and current literature on subjects covered in this study including mineral nutrition, the social honey bee, salt taste and behavioural regulation of nutrient intake. That being said, I aim to restate the current gap in the literature and the significance of studying the honey bee mineral taste preferences and regulation of intake. Next, I emphasize which experimental approach I employed in the following Chapters to tackled this gap in knowledge. Last, I provide a road map indicating the overal structure of this thesis and what to expect from the following Chapters.

1.2 Animal Nutrition – The Necessary Intake of Nutrients

Every living organism eats. Nutrition is a complex biological process involving food intake, digestion, absorption, assimilation, metabolism and excretion that supports development, growth, reproduction, health and survival. Food intake is a behaviour observed in all animals whereby they obtain chemical elements (nutrients)

from food and, thus is the first route towards nutrition (Berdanier and Zempleni, 2009; Simpson and Raubenheimer, 2011). Nutrients are dietary chemical compounds, organic or inorganic, required to meet biological needs. Nutrients can be essential or non-essential: a nutrient is considered essential if it is required but cannot be synthesized internally, and thus must be acquired externally (Berdanier and Zempleni, 2009). In contrast, a non-essential nutrient is any food-derived component, like dietary fiber (polysaccharide), that mediates the digestibility and absorption of essential nutrients, and others that can be produced after essential nutrients (e.g. cysteine) (Anderson et al., 2009). Unlike mammals, insects require a dietary source of sterols during development (Hobson, 1935). The essentiality of nutrients is primarily based on the observation that if in absence of that nutrient the adequate development, growth and reproduction is impaired (Mertz, 2009). For example, in locusts, the absence of ascorbic acid reduces food acceptability, resulting in the reduction of feeding and, thus, poor growth (Dadd, 1960). Therefore, ascorbic acid is an essential nutrient for locusts. In colourful birds carotenoids, a plant-derived pigment that they cannot synthetize, are required for visual sensitivity, signalling and mating success (Toomey and McGraw, 2009; Senar *et al.*, 2010). Nevertheless, nutrient essentiality is not only a matter of presence/absence, but also about the amount that is consumed and the relative proportion between different nutrients. Essential nutrients can be classified into two groups: macronutrients or micronutrients. Macronutrients are organic compounds required in larger amounts (e.g. mg or g/Kg/day), provide structure and fuel the main metabolic functions. Micronutrients, in contrast, are non-caloric nutrients (mineral salts and vitamins) that are required in smaller amounts (mg, μg/Kg/day or lower) supporting metabolism in function and regulation (Berdanier and Zempleni, 2009). Water is also an essential and critical nutrient as it provides the supporting medium in which all the vital metabolic reactions take place (Berdanier and Zempleni, 2009; Jéquier and Constant, 2010; Cohen, 2015).

In overal, nutrients are the chemical factors that drive the interactions between an animal's physiology, behaviour, habitat and ecology (Simpson and Raubenheimer, 2011).

1.3 Macronutrients: Function and Basic Requirements

Macronutrients comprise carbohydrates, proteins and lipids and support the main structural and metabolic functions. Dietary carbohydrates (sugars, starch) can provide up to 60% of daily energy intake in humans, and are the primary fuel to support flight in some birds (e.g. hummingbirds) and flying insects (e.g. bees and locusts) (Beenakkers, 1969; Chen and Welch, 2014). Though, caloric carbohydrates (e.g. sucrose) are required in substantial amounts, their essentiality is controversial in human nutrition, as they can be generated *de novo* as a product of fat metabolism and gluconeogenesis (Nelson and Cox, 2013). But for flying insects, particularly for bees, the sugar turnover is not fast enough during flight (Blatt and Roces, 2001), rendering ingestion of sugars extremelly necessary to support high energetic demands (Suarez, 2005). Digestible proteins and the ten essential amino acids provide structural and metabolic substrates (enzymes, cellular messengers, carriers) (Berdanier and Zempleni, 2009). The requirement for protein is the minimum intake sufficient to sustain metabolic demands and growth rates. In adult bees, ingesting too much protein or essential amino acids negativelly affects survival (Altaye *et al.*, 2010; Pirk *et al.*, 2010; Paoli, Donley, *et al.*, 2014). Dietary lipids, compared to carbohydrates, have greater energy value per gram, and provide essential fatty acids. Lipids are insoluble in water, facilitate the absorption of other lipid-soluble nutrients (e.g. vitamin A), are integral part of cellular membranes (as phospholipids) and can function as systemic messengers (hormones) (Berdanier and Zempleni, 2009; FAO, 2010). In bees, for example, deficiency in omega-3 (fatty acid) affects learning (Arien *et al.*, 2015).

1.4 Micronutrients: Function and Basic Requirements

Micronutrients (vitamins and mineral salts) demonstrate a diverse biochemical function (enzyme cofactors, macronutrient metabolism, hormone-like functions). Virtually, all essential micronutrients are involved in energy metabolism (Huskisson, Maggini and Ruf, 2007), and some are important for immune function (e.g. ascorbic acid, iron, zinc and selenium) in both mammals (Wellinghausen, Kirchner and Rink, 1997; Maggini et al., 2007; Yatoo et al., 2013) and insects (Popham, Shelby and Popham, 2005; Popham and Shelby, 2009; Cohen, 2015). Their roles and requirements are well known for humans and livestock (Underwood, 1971; FAO *et al.*, 1998; Soetan, Olaiya and Oyewole, 2010), but not so well for other animals. For example, vitamin E (α-tocopherol), folate and zinc modulate gene expression (Beckett et al., 2014); vitamin C (ascorbic acid) and E are known as potent non-enzymatic antioxidants. These vitamins can directly quench reactive oxygen species, and trace metals participate as cofactors in enzymatic antioxidant defence mechanisms (FAO *et al.*, 1998; Zhang *et al.*, 2015). Recently, dietary Zn has been implicated in boosting antioxidant activity in honey bees (Zhang *et al.*, 2015). Also, compared to mammals, insects require much lower amounts of calcium (Ca), iron (Fe), sodium (Na), but more potassium (K) (Cohen, 2015). Few reports exist on the role of mineral salts in insect nutrition and requirements, and how mineral ingestion affects the life history of an insect. Because mineral requirements are low in quantitiy, they are often assumed to be met by default when ingested in food. However, it is likely that every species may present nutritional specificities and thus generalizations of nutritional requirements across insect species can be challenging (House, 1962).

1.4.1 Minerals as Essential Nutrients

Mineral salts cannot be biosynthesized, thus, to fulfil nutrient requirements for proper metabolic functions, animals must acquire these from food. As such, minerals are micronutrients with emphasis in animal metabolism and several other biological

processes (e.g. tissue structure; to defence against pathogens.) For example, Ca recruits antioxidant enzymes via intracellular signalling (Krautz, Arefin and Theopold, 2014), and mediates haemolymph coagulation in physically injured insects (Bidla *et al.*, 2005). Others such as Zn, Mn, Cu, Fe are required in antibacterial defence (Locke and Nichol, 1992; Dunphy, Niven and Chadwick, 2002; Zhang *et al.*, 2015). Fe assumes a pivotal role in both insect metabolism (Nichol, Law and Winzerling, 2002) and energetic metabolism of flying insects (e.g. cytochrome C oxidase, NADPH oxidase). Zn, Cu and Mn are involved in the formation and hardness of insect cuticle, thus contributing to insect external structure (Lichtenegger *et al.*, 2003; Schofield *et al.*, 2003). Minerals can be subdivided into three classes according to bulk requirements: *macro* (Ca, K, Mg, Na, S, P, Cl), which tend to be required in $mgKg^{-1}$, whereas *micro* (Fe, Zn, Mn, Cu) and *trace* (Se, Co, Cr, Ni) elements are necessary in μgKg^{-1} or below (Hidiroglou, 1982; Berdanier and Zempleni, 2009). Mineral requirements are usually expressed in parts per million (ppm) or per billion (ppb). Though Fe, Zn, Mn and Cu occur in low sometimes trace amounts, are essential nutrients for both mammals and insects. Other *trace* elements (e.g. Cd, Pb, Hg, Al) can co-occur, but are readily toxic even if ingested in vestigial doses (Wuana and Okieimen, 2011; Formicki *et al.*, 2013; Meindl and Ashman, 2013; Exley, Rotheray and Goulson, 2015). Both *micro* and *trace* elements display high density properties comprising together the transition metals category in the periodic table (Williams, 1971). For this reason, these metals are frequently termed as heavy metals altogether. Nonetheless, this terminology is controversial and should be avoided otherwise (Pourret and Bollinger, 2018). Mineral requirements frequently refer to a specific element (e.g. Ca, K, Mg, Fe), though they exist as part of molecules such as NaCl. For this reason, minerals often referred to as salts. At the chemical level, mineral salts have the property to undergo a neutralization reaction to generate an electrically neutral product: one cation (positively charged ion) and one anion (negatively charged ion) (Williams, 1971). This occurs when dissolved in water and, therefore, these compounds have the ability to conduct electricity when an electrical potential is applied (Williams, 1971).

Because of this property, minerals can be termed electrolytes or ionic compounds, which can be either organic (CH_3CO^{2-}) or inorganic (Cl^-, K^+, Na^+, Mg^{2+}, PO_4^{2-}). To prevent misconceptions, hereafter, I will refer to **minerals** or **minerals salts** in general. In cases where I intend to specify a mineral category either *macro* or *micro* elements, I will use the term **salts** for Na, K, Mg, Ca, or **metals** for Fe, Zn, Cu, Mn. In this study, I focused in minerals of inorganic origin. This work will cover eight dietary minerals recognized as essential for most insects (House, 1962; Dadd, 1973) and honey bees included (Haydak, 1970; Brodschneider and Crailsheim, 2010).

The physiology of inorganic metabolites, factors affecting its absorption and routes of excretion have been extensively studied in both insects and mammals; for review see (Hidiroglou, 1982; Kerkut and Gilbert, 1985; Berdanier, Corny and Yousef, 2009; Tercilia Vilela de Azeredo, 2014). The function and importance of essential salts and metals are conserved at the cellular level from mammals to insects. However, its routes of excretion can be distinct. In contrast to mammals that produce urine, insects tend to excrete nitrogenous wastes as semi to dry feces (Kerkut and Gilbert, 1985). Table 1.1 (for salts) and 1.2 (for metals) indicate the main metabolic functions of relevant minerals, factors affecting absorption and a brief comparison of its routes of excretion between mammals and insects.

Mineral excretion and the physiological mechanisms underlying postingestive regulation of minerals have been well-addressed in contrast to the mechanisms regulating mineral salt intake in insects. This is especially true for metals. If a mineral is to be regulated from intake to excretion, physiological mechanisms should be tuned to adjust the rate of intake in first place.

Table 1.1 Major salts (inorganic nutrients) in insect nutrition and covered in this study. Principal metabolic functions, absorption and main routes of excretion‡ adapted from (Cohen, 2015) for insects and from (Berdanier, Corny and Yousef, 2009) for humans.

Macro mineral‡‡	**Ion**	**Type**	**Main function and importance** (in insects and humans)	**Absorption** (in humans and possibly insects)	**Excretion‡‡‡** (in humans)
SALTS					
Potassium	K^+	Alkali metal	Major intracellular electrolyte; excitable tissues; cellular membrane function; water balance and osmotic pressure along with sodium (Na^+) and chloride (Cl^-); Na/K pump	Readily absorbed	Urine
Sodium	Na^+	Alkali metal	Critical in excitable tissues; cellular function and neuronal transmission; water balance and osmotic pressure along with potassium (K^+) and chloride (Cl^-); Na/K pump	Readily absorbed	Urine
Calcium	Ca^{2+}	Alkali metal	Metabolic regulation as intracellular signalling; e.g. Ca recruits antioxidant enzymes (e.g. H_2O_2); muscular excitation; mineralization of insect cuticle.	Poor absorption; facilitated in the presence of vitamin D; reduced by phytates, oxalates, excess of dietary protein; tannins and presence of Zn, Mn or Cu	Faeces
Magnesium	Mg^{2+}	Alkali metal	Involved in carbohydrates metabolism (glycolysis); enzyme co-factor in phosphorylation reactions (ATP); participates in neuro-muscular transmission; main intracellular divalent ion;	Passive diffusion and active transport; facilitated in the presence of vitamin D; reduced in the absence of Ca and vitamin D, and phytates	Urine
Chloride‡‡‡	Cl^-	Halogen	Universally required by all organisms; enzyme co-factor; involved in the maintenance of membrane potential (electrical charge), which is key part of excitable tissues and cells in muscle and nerve tissues.	Readily absorbed via Na-dependent mechanism	Urine

‡ Mineral functions, absorption and excretion routes are indicated but are not a subjected specifically covered in this study.

‡‡ Major and minor elements refer to the average amount required.

‡‡‡ Note that nitrogenous wastes in terrestrial insects are mostly semi to dry faeces/frass in the form of uric acid not urinary urea.

‡‡‡‡ Chloride is represented here because is the main anion (negatively charged ion) required in insects and was the conjugated anion for salts/metals used here.

Table 1.2 Major metals (inorganic nutrients) in insect nutrition and covered in this study. Principal metabolic functions, absorption and main routes of excretion‡ adapted from (Cohen, 2015) for insects and from (Berdanier, Corny and Yousef, 2009) for humans.

Micro mineral‡‡	**Ion**	**Type**	**Main function and importance** (in insects and humans)	**Absorption** (in humans and possibly insects)	**Excretion**‡‡‡ (in humans)
METALS					
Iron	$Fe^{2+/3+}$	Transition Metal	Involved in immune function; enzyme-cofactor; key player in aerobic metabolism; toxin degradation metabolism (cyt P450); purine metabolism (production of nitrogenous waste products); precursor of moulting hormone (an ecdysis hormone)	Poor absorption; Only ferrous (Fe^{2+}) iron is absorbed in the gut epithelia; Facilitated in the presence of vitamin C and Cu; Reduced by phytates, Zn; low dietary proteins	Faeces
Zinc	Zn^{2+}	Transition Metal 1	Enzyme co-factor involved in antioxidant capacity (SOD); possibly involved in proper functioning of taste; Part of zinc-finger proteins (DNA-binding); Metalothionine transcription; impact activity of DNA/RNA polymerase; mineralization of insect cuticle	Poor absorption; Facilitated by dietary proteins and vitamin D; Reduced by excess of Cu, Mn, Fe; phytates	Faeces
Manganese	Mn^{2+}	Transition Metal	Enzyme co-factor; involved in lipid metabolism; essential to reactions of ATP/UTP; mineralization of insect cuticle	Poor absorption	Faeces
Copper	Cu^{2+}	Transition Metal	Enzyme co-factor; involved in iron metabolism and antioxidant capacity (SOD); mineralization of insect cuticle	Poor absorption; Reduced by excess zinc and phytates	Urine

‡ Mineral functions, absorption and excretion routes are indicated but are not a subjected specifically covered in this study.

‡‡ Major and minor elements refer to the average amount required.

‡‡‡ Note that nitrogenous wastes in terrestrial insects are mostly semi to dry faeces/frass in the form of uric acid not urinary urea.

1.5 Introducing the Honey Bee, *Apis mellifera*

1.5.1 Colony Structure of a Superorganism

Bees constitute a group of approximately 20,000 bee species (Ascher and Pickering, 2016), standing out as the most relevant insect pollinators among butterflies, ants and moths. Honey bees belong to the Hymenoptera order (Family: Apidae) and live in densely populated colonies of about 50,000 closely related individuals during summer, and approximately 20,000 individuals in winter time (Winston, 1987). Honey bees are one of the few representatives of sociality among bees (Heinrich, 1975). Their high social organization (eusocial) demonstrates three key features: 1) reproductive division of labour (different behavioural groups of individuals or castes); 2) overlapping generations and 3) cooperative brood and colony care (Wilson, 1971; Wilson and Hölldobler, 2005). Eusocial insects differ from other levels of sociality, as some castes lose the ability of performing other group tasks. Their colony structure is largely comprised of adult females, one reproductive queen responsible for laying up 2,000 of eggs per day and thousands of functionally sterile worker bees that are central in the colony maintenance and nutrition (Brodschneider and Crailsheim, 2010). Few thousands of drones (male fertile bees) are also reared to mate virgin bee queens during the spring/summer months. Depending of the caste, honey bees demonstrate different developing times: queen bee (16 days); worker bee (21 days); or drone (24 days), all moving through the same four basic stages: egg, larvae, pupa and adult. During the year, bees show two main ways of organization: in the spring and summer, division of labour is used to boost growth rate and food resources storage; in winter time, the main task is to ensure worker survival, thus bees become generalists (Figure 1.1 a and b).

As part of long-lived colonies, bees collect, process and store food inside the hive to prevent starvation in dearth periods (Winston, 1987). Adult workers process nectar and pollen into storage products, which are maintained inside capped comb

cells. They also produce two other products: beeswax and propolis. Beeswax is a yellow-coloured natural product produced by abdominal glands (ventral place segments – sternites) (Snodgrass, 1956; Graham, 2010). It is mostly composed of esters of fatty acids and used inside the hive for wax comb building. Inside these cells, young bees are raised or food reserves are capped and stored. In contrast, propolis is a dark-brown product ("bee glue") and is a resinous substance produced by mixing with saliva and beeswax. Bees use propolis as a sealant to repair the hive (Graham, 2010). Wheeler (Wheeler, 1911) first coined social insects as superorganisms, meaning that each individual contributes differently to the general good and survival of the whole colony. They function as a social unit. Such individuals may not survive alone for extended periods of time. They work synergistically for the self and the collective well-being. Nutrition in social insects, honey bees inclusive, has a fundamental role in caste determination and behavioural plasticity.

1.5.2 Caste Differentiation

Distinct feeding regimes (diet quality and quantity) control not only caste development and reproductive differentiation (queen or worker) (Kucharski *et al.*, 2008; Lockett, Kucharski and Maleszka, 2012), but also behavioural plasticity between adult workers (nurses to foragers) (Toth and Robinson, 2005). Caste determination during larval stages is independent of genetic difference as all larvae are fed the same type of protein-rich jelly produced in head glands of young nurse bees in the first 3 days. Then and onwards, while queen-destined larvae are kept in that same rich jelly (royal jelly, RJ) (Kucharski *et al.*, 2008; Kamakura, 2011), worker-destined larvae are fed a simpler diet consisting of a mixture of pollen, sugars and few glandular secretions (Winston, 1987; Brodschneider and Crailsheim, 2010), see review (Maleszka, 2018). This shift in larval diet dictates the reproductive capacity of female bees. Among, female worker castes there is a remarkable behavioural plasticity, often termed division of labour, which is characterized by temporal

polyethism (Seeley, 1982; Calderone, 1995; Beshers *et al.*, 2001; Toth and Robinson, 2005; Ament, Wang and Robinson, 2010; Johnson, 2010; Amdam, 2011; Herb *et al.*, 2012). Worker bees transit from behavioural activities as they age and as they change diet regimes (Winston, 1987; Crailsheim *et al.*, 1992), see (Figure 1.1 a and b). Adult worker maturity relates to task transitioning. As young bees (nurses) age, other tasks are performed inside the hive (e.g. cleaning up, building, guarding) and, finally, foraging (Figure 1.1 a).

Division of labour and behavioural maturation of adult workers is not static; it occurs at different rates and is influenced by genetic backgrounds (Calderone, 1995; Pankiw and Page, 2001; Amdam *et al.*, 2006; Page and Amdam, 2007; Wang *et al.*, 2010; Siegel *et al.*, 2013). Worker bees are organized in such a way that a specific group of older bees (foragers) is allocated the task of finding, selecting and collecting food outside the hive; the others perform in-hive tasks (nursing, comb building, food processing). Therefore, foraging and nursing are vital behaviours for each individual and colony nutrition (Figure 1.1 c). Honey supplies most of adult workers' energetic demands, whereas pollen is virtually the exclusive source of non-carbohydrates necessary for reproducing females and developing larvae. Pollen is gathered and transported by forager bees, then consumed and digested by young nurse bees, which supports the production of glandular jelly for feeding both larvae, the queen and young workers (Crailsheim *et al.*, 1992). Young nurse bees (0-12 days old) are pivotal in mediating colony nutrition by both producing nutrient-rich jelly (Crailsheim *et al.*, 1992; Hrassnigg and Crailsheim, 1998) and distributing the digested nutrients among nestmates (Brodschneider et al., 2017) via trophallaxis (food sharing mouth to mouth) (Crailsheim, 1998). The interplay between nursing and foraging is, therefore, critical in the regulation of food selection and feeding (Figure 1.1. c).

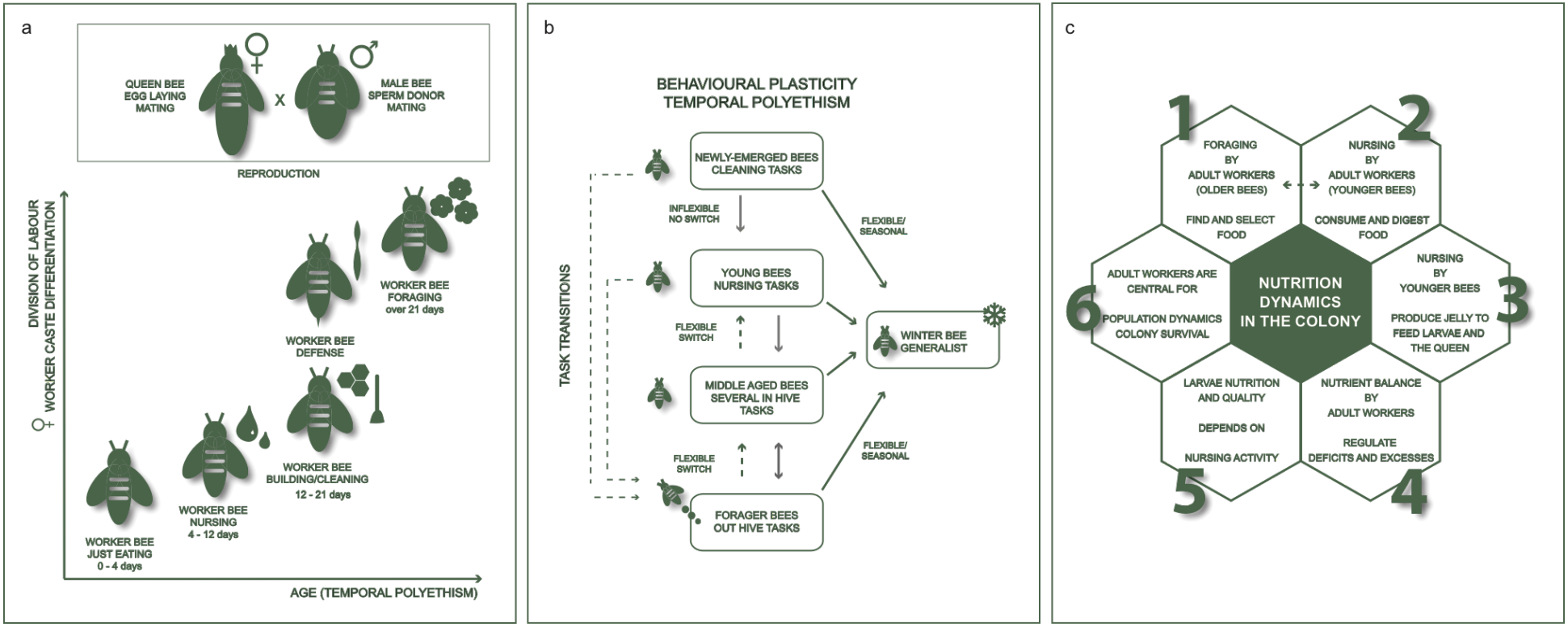

Figure 1.1 Honey bee caste system and division of labour. **a)** Bee hive caste composition. **b)** "Push-pull" model. Bees show temporal polyethism in spring and summer and are generalist workers in winter. Solid arrows indicate natural caste transitions; dashed arrows show atypical caste transitions. Young bees are recruited from their tasks by the emergence of newly emerged bees; middle-aged bees are pulled towards foraging behaviour via interactions with older bees. All workers are assumed to winter in similar state. This model was reviewed by (Johnson, 2010) and proposed by (Seeley, 1982; Calderone and Page, 1988; Calderone, 1995; Toth and Robinson, 2005; Amdam, 2011). **c)** The behavioural dynamics demonstrate the pivotal role of adult workers nurses (feeding) and foragers (food gathering) and their feeding behaviours on securing colony survival (adapted from review paper (Brodschneider and Crailsheim, 2010).

1.5.3 Behavioural Maturation and Assessment of Colony Needs

Dynamics involving food selection and nourishment among social insects can be far more complex. Newly emerged bees build up their body composition up to day 6 by consuming approximately 80% of stored beebread required for nursing behaviour (Crailsheim, 1990; Crailsheim *et al.*, 1992). Then as worker bees age (9–16 days old) and become foragers, they shift their feeding habits towards carbohydrates. As a result, pollen consumption decreases, HPG in the head shrink and both proteolytic activity and digestibility of proteins decrease (Crailsheim, 1990; Crailsheim *et al.*, 1992). In foragers, task specialization involves not only a genetic component (Page, Erber and Fondrk, 1998), but also translates into different gustatory sensitives (Pankiw and Page, 2000; Scheiner, Page and Erber, 2001). Foragers specialized in water, nectar or pollen collection tend to demonstrate different sensitivities to sucrose concentrations. Classical Proboscis Extension Reflex (PER) studies revealed that forager bees highly responsive to low sucrose concentrations (e.g. 0.1%) are also more responsive to stimuli in other sensory modalities (e.g. salts) and likely to collect pollen instead of nectar (Pankiw and Page, 2000; Scheiner, Page and Erber, 2001); for a review see (Scheiner, 2004). Transition from nursing to foraging in adult workers not only follows a change in behaviour and diet (towards more carbohydrates), but is also accompanied by further physiological changes (Robinson, 2002; Amdam and Omholt, 2003; Nelson *et al.*, 2007). Transition to foraging can be influenced by nutritional status, hormonal feedback, and larval pheromone cues. For example, at the onset of foraging, abdominal lipid stores of forager bees drop significantly (Toth and Robinson, 2005; Toth *et al.*, 2005; Wang *et al.*, 2010). This transition seems to be mediated by a hormonal feedback loop between levels of juvenile hormone (JH) and vitellogenin (Vg, egg-yolk precursor). For example, hemolymph titres of JH and Vg are recognized to be inversely present depending on whether a bee is in a nursing state (low JH and high Vg) or in a foraging state (high JH and low Vg) (Amdam and

Omholt, 2003; Ihle *et al.*, 2010; Nunes *et al.*, 2013). Also, brood released pheromones have been indicated to affect division of labour and adult workers physiology (Le Conte, Mohammedi and Robinson, 2001). The presence of larvae can impact nursing (e.g. nurse feeding responses) and foraging (e.g. collection of nutritional resources) behaviours through pheromone cues and, thus, can affect colony composition, the quality of food stores and colony fitness (Sagili and Pankiw, 2009; Traynor *et al.*, 2017). For a review on honey bee social organization and regulatory mechanisms of sociality see (Page, 2013). Altogether, these studies demonstrate the complexity of honey bee social biology and how nutrient cues affect significantly features such as morphology, physiology, behavioural and colony structure.

1.5.4 Delivering Pollination Services

Herbivores or phytophagous insects show different feeding habits, among them are biting, chewing, sucking and lapping such as in honey bees. Within the vast group of insects, butterflies (Lepidoptera) and bees (Hymenoptera) are specialists in floral feeding (Browne, 1975; Nicolson, Nepi and Pacini, 2007). Honey bees are generalist feeders, foraging on a wide range of flower species, and they excel other bees in flower constancy (Grant, 1950; Free, 1970; Grüter *et al.*, 2011), which means that during one foraging trip, bees from the same colony are likely to visit the same flower species, bypassing other potentially rewarding flowers. This behaviour supports the mutualistic relationship between honey bees and flowering plants. By foraging on flowers, nectar-feeding insects act as effective biogenic agents of pollination. Pollination is one route for floral plants fertilization. It involves the transfer of pollen grains (precursors of male gametes) from the stamen (filament + anther = male part of a flower) to the pistil (stigma + style + ovary = female part of the flower) of the same species, and is performed by animal pollinators (Faegri and Van der Pijl, 1979).

From the service provided by both wild and managed pollinators, the revenue for our food production system is immense (Garibaldi *et al.*, 2013). Insect pollination

services supply the agricultural and food-related economy generating up to US$15.12 billion in the US agricultural industry (Calderone, 2012), and contributing to ~9.5% of the total value of the production of human food globally (€153 billion) (Gallai *et al.*, 2009). Among insect pollinators, bees forage on more than 90% of the major global crops (Klein *et al.*, 2007), rendering honey bees the leading managed pollinator worldwide in large cropping systems.

1.6 Source of Nutrients – Honey Bee Food

Honey bees evolved to feed exclusively on flowering plants (Angiosperms). They are generalist pollinators that forage on floral nectar and pollen. Bees collect food to feed their colony and young, and, in return, plants have their pollen widespread from one flower to another of the same species, and their reproduction ensured. Both nectar and pollen constitute the prime food sources that bees are able to digest, and its composition is greatly dependent on factors such as soil composition and geographical location (Black, 2006; Ball, 2007; Campos *et al.*, 2008).

1.6.1 Nectar – Energy Food Source

Floral nectar is a sugar-rich semi-viscous liquid with amino acids and other trace compounds produced by flowering plants in specialized glands termed nectaries (Nicolson, Nepi and Pacini, 2007). These glands are displayed in female reproductive parts (ovary, calyx, stamen, corolla) and provide a sugar reward for visiting pollinators that collect pollen (Nicolson, 2011). Nectar composition varies widely, but is mostly composed of digestible carbohydrates (e.g. sucrose, glucose and fructose) with up to 60% of total sugars, which fuel flight (Suarez, 2005; Nicolson, 2011; Hendriksma, Oxman and Shafir, 2014). Sugars present in nectar have been reported to differently influence learning and memory abilities of forager bees (Simcock, Gray, *et al.*, 2017). Protein and especially amino acids are also present in nectar and can influence honey bee gustatory responses (Simcock, Gray and Wright,

2014). Interestingly, proline is a non-essential amino acid but commonly preferred and used by bees to support the onset of flight (Micheu, Crailsheim and Leonhard, 2000). Lipids may also be present in nectar, but rather in trace amounts. Minerals in nectar vary greatly especially in quantity as depicted in Table 1.3. Yet, floral nectars often collected by honey bees do not contain high concentrations of Na (~10 mM; 230 ppm Na^+) (Nicolson and Worswick, 1990). High mineral nectar contents seem to deter bees from feeding (Waller, Carpenter and Ziehl, 1972; Afik, Dag and Shafir, 2008; Afik et al., 2014) and, therefore, seem to act as regulators of flower visitation. Nectars such as avocado are not typically collected by honey bees. Avocado nectar contains low Na (54 ppm), but rather high K (> 3,000 ppm) and P (> 600 ppm) contents, which may explain nectar rejection by bees (Afik *et al.*, 2006, 2009, 2014). Little is yet known about detection, gustation of metals in nectar and how it influences bee behaviour. For example, selenium (1.5 ppm) did not deter flower visitation (Hladun, Parker, *et al.*, 2013). In bumblebees different metals in nectar induced divergent effect on foraging behaviour, aluminium (Al) did not affect flower visitation, but nickel (Ni) reduced the time spent on foraging (Meindl and Ashman, 2013). Secondary compounds are also present in nectar in trace quantities but depending on chemical identity and concentration, they may induce a range of effects; for review see (Stevenson, Nicolson and Wright, 2017). Ingestion of artificial or natural liquid diets exhibiting high mineral contents have been reported to induce malaise-related symptoms and to decrease adult workers' lifesapn (Herbert, 1979; Horn, 1985; Imdorf *et al.*, 1985; Crailsheim and Pabst, 1988; Horr, 1998; Cohen, 2015).

Table 1.3 General review of mineral composition of nectar/honey in different locations and flower samples, and comparison with royal/worker jelly and bee-collected water. Mean ranges or mean contents are presented in mg/Kg in fresh weight (FW).

Mineral Components [mg/Kg FW]	*Arbutus unedo* nectar[1] (mean)	Natural honey[2] (range)	Wild / orchard honeys[3] (mean)	Honey (range)[4]	*Persea americana* nectar / honey[5] (mean)	*Citrus* sp. nectar[5] (mean)	*Persea americana* nectar[6] (mean)	*Persea americana* nectar[7] (male / female flowers) (mean)	Broad range in nectar/ honey (estimation)	Royal Jelly[8] (range)	Worker Jelly[8] (range)	Fresh*/tap water[9]
Macro elements												
Calcium	370.	22.9–72.8	33.5/37.6	23.0–68.0	<150./82.7	<150.	198.	248./164.	20.0–400.	109.–276.	148.–247.	–
Magnesium	69.0	5.50–145.	16.4/21.7	11.0–56.0	188./205.	<5.00	158.	21.0/163.	<5.00–250.	305.–436.	147.–343.	30.0/27.0
Potassium	88.0	166.–736.	406./425.	100.–588.	3,946./3,768.	185.	1,724.	4,555./4,534.	80.0–5,000.	1,938.–2,852.	1,050.–2,127.	10.0/6.00
Sodium	253.	7.80–74.1	12.3/18.3	6.00–35.0	53.8/58.9	18.5	52.3	34.8/39.3	5.00–80.0	170.–690.	134.–689.	130./104.
Phosphorous	–	35.7–699.	–	23.0–50.0	511./652.	18.5	302.	568./644.	15.0–700.	–	–	0.30/0.00
Sulfur	–	–	–	–	170./188.	<5.00	116.	145./139.	<5.00–200.	–	–	–
Chloride	–	–	79.3/92.8	–	–	–	–	–	–	–	–	–
Micro elements												
Iron	10.0	0.80–6.70	14.2/3.07	1.20–4.80	13.5/9.30	<5.00	–	–	0.50–15.0	23.7–39.0	13.2–30.7	–
Zinc	5.00	0.50–8.40	0.41/0.90	–	<30.0/10.9	<30.0	–	16.8/16.5	0.50–30.0	17.6–24.9	9.75 – 17.5	–
Manganese	–	0.10–4.70	0.83/0.64	0.17–0.44	–	–	–	7.10/4.80	0.10–10.0	0.52–2.48	1.49 – 2.93	–
Copper	2.00	0.10 – 2.20	0.41/0.74	0.14–0.70	3.10/3.20	<0.50	5.80	3.70/2.70	0.10–6.00	3.67–4.74	1.43 – 3.88	–
Trace elements												
Selenium	–	–	–	–	–	–	–	–	–	–	–	–
Boron	–	–	–	–	10.8/9.90	4.20	–	17.6/12.8	–	–	–	–
Other												
Total matter (% dry)	17.1	–	–	–	–	–	30.0	37.0/26.0	–	–	–	–
Total Ash (%)	0.48	–	–	–	–	–	–	–	–	–	–	–
Total Sugar (%)	16.5	–	–	–	–	–	–	–	–	–	–	–
Total Protein (g/100g FW)	–	–	–	–	–	–	–	–	–	11.2–14.1	13.4–17.9	–
Moisture (g/100g FW)	–	–	–	–	–	–	–	–	–	57.4–73.2	49.0–61.8	–

[1]Southern France (Rasmont *et al.*, 2005); [2]Poland (Grembecka and Szefer, 2013); [3]Portugal (Almeida-Silva *et al.*, 2011); [4]USA (Ball, 2007); [5]Israel (Afik *et al.*, 2006); [6]Israel (Afik, Dag and Shafir, 2007); [7]Mexico (Afik *et al.*, 2014); [8]China (Wang *et al.*, 2016); [9]USA (Lau and Nieh, 2016). *bee-collected water.

Nectar foragers returning to the hive pass on nectar stored in the crop (or honey sack, a specialized part of the foregut) to receiver bees that, through repeated regurgitation steps and fanning, promote water evaporation and convert nectar into honey. Adult honey bees can satisfy their energetic dietary requirements by consuming nectar/honey (Haydak, 1970; Brodschneider and Crailsheim, 2010). As such, honey, derived from floral nectar, is the main energy source (carbohydrates), whilst pollen, derived from floral pollen, provides most of other essential nutrients (Dadd, 1973; Brodschneider and Crailsheim, 2010).

1.6.2 Pollen – Prime Source for Non-Carbohydrates

Pollen nutrition is vital for colony survival as pollen shortages decrease growth, lifespan and immune capabilities of honey bees (Standifer, 1967; Knox, Shimanuki and Herbert, 1971; Schmidt, Thoenes and Levin, 1987; Crailsheim, 1990; DeGrandi-Hoffman *et al.*, 2010; Alaux *et al.*, 2011; Huang, 2012; Di Pasquale *et al.*, 2013; Brunner *et al.*, 2014; Smart *et al.*, 2016).

Bee-collected pollen is another floral reward, occurring in the male parts of flowering plants as single grains which, upon germination, produce male gametes (Nicolson, 2011). In contrast to nectar, pollen is high in protein, amino acids, lipids, vitamins and minerals. Therefore, pollen is virtually the exclusive source of non-carbohydrate nutrients for bees (Brodschneider and Crailsheim, 2010). Pollen is collected from flowers and mixed with salivary secretions and nectar to agglutinate single pollen grains into one whole pellet (pollen baskets) (Graham, 2010; Nicolson, 2011). Then, forager bees load these pellets on specialized appendages on the hind legs (corbicula) (Snodgrass, 1956) for secure transport back to the hive. Inside the hive, pollen is transformed into beebread, which is a mixture of pollen, honey and glandular secretions (Nicolson, 2011). Pollen is consumed and digested by young nurse bees to support the production of glandular jelly for feeding both larvae, the queen and young workers (Crailsheim *et al.*, 1992). Honey and beebread are stowed

in wax cells inside the hive and function as food stores for colony growth and survival during winter and unfavourable conditions.

Pollen composition is largely variable (2–60%) depending on the season and floral origins (Roulston and Cane, 2000). High crude protein pollen content has been used as a proxy for high chemical quality pollen and nutritional value, by means of protein digestibility (Crailsheim *et al.*, 1992) and amino acid composition that may impact adult health (De Groot, 1953; Cook *et al.*, 2003; Nicolson and Human, 2013; Paoli, Donley, *et al.*, 2014; Arganda *et al.*, 2017). Not only proteins, but also lipids in pollen can affect health and fitness in bees (Arien *et al.*, 2015; Vaudo *et al.*, 2017). Lipids are found in pollen (1–18%) (Roulston and Cane, 2000) among which 24-methylene cholesterol is one of the most important to complete development into adulthood (Haydak, 1970; Herbert *et al.*, 1980; Svoboda *et al.*, 1982; Brodschneider and Crailsheim, 2010; Huang, 2012). Though the nutritional value of pollen is often assessed by the amounts and type of proteins, pollen is the major source for mineral salts. Major minerals found in pollen include K, P, S, Mg, Ca, Na, Fe, Zn, Cu and Mn (Somerville and Nicol, 2002; Campos *et al.*, 2008; Morgano *et al.*, 2012; Filipiak *et al.*, 2017); see also (Black, 2006b). These micronutrients are important for osmoregulation (Nicolson and Worswick, 1990) and sustain metabolic and tissue functions. They act as cofactors of metalloenzymes (~1/3 of all enzymes known so far) (Hoppert, 2011) and metal-responsive transcription factors (Günther, Lindert and Schaffner, 2012); for a review in insects see also (Dow, 2017). Pollen contains between 1 to 7% of minerals salts; this range of values is also found in other plant tissues (Lunden, 1954; Atkins, Grout and Dadant & Sons, 1975; Herbert, 1992); see Table 1.4 for an overview of minerals ranges in bee-collected pollen reported in the literature. Some pollen types may even contain excess of these salts and become toxic to bees (Herbert and Shimanuki, 1978c). K is the most abundant element present (> 50% of total ash) and also the most required by insects (greater than for mammals and birds), followed by Mg (~20%), Ca and Na (~10%) and all the other elements (~10%) (Standifer, 1993; Somerville, 2005; Campos *et al.*, 2008; Brodschneider and Crailsheim, 2010).

Field studies demonstrated that colonies increased foraging range to compensate low pollen diversity (e.g. landscape) and maintain both the amount and diversity of pollen collected (Danner et al., 2017); pollen diversity improves adult bee health, physiology, immune function and survival (Alaux *et al.*, 2010, 2017; Di Pasquale *et al.*, 2013; Frias, Barbosa and Lourenço, 2016), and secures colony survival (Smart *et al.*, 2016). Nevertheless, polyfloral blends are not necessarily better than some monofloral pollen types of good nutritional values (e.g. *Rubus* and *Prunus* pollen) (Somerville, 2001; Di Pasquale *et al.*, 2013; Filipiak *et al.*, 2017). Secondary components in pollen (e.g. *p*-coumaric acid) have been also reported to improve detoxification mechanisms in the honey bee gut (Mao, Schuler and Berenbaum, 2013).

Pollen is the major source of micronutrients for bee nutrition. Besides, minerals in pollen serve are phagostimulants that can, for example, interact with other nutrients in food and affect food acceptability (palatability) in both mammals and insects (Dethier, 1977; Trumper and Simpson, 1993; Schmidt *et al.*, 1995; Breslin and Beauchamp, 1996). Palatability of a nutrient in diet is associated with acceptability or rejection of food. The ability to taste and to accept it will drive the appropriate feeding response and, ultimately nutrition (Dethier, 1976; Cohen, 2015). Yet, it is still controversial whether honey bees can detect and discriminate nutrients in pollen through gustatory cues (Pernal and Currie, 2002; Leonhardt and Blüthgen, 2012; Ruedenauer, Spaethe and Leonhardt, 2015; Corby-Harris *et al.*, 2018).

Table 1.4 General review of mineral composition of bee-collected pollen in different locations. Mean ranges or mean contents are presented in mg/Kg in dry weight (DW).

Mineral Components [mg/Kg DW]	*A. unedo* Ref[1] (mean)	Ref[1] (mean)	Ref[2] (range)	*E. wandoo*[3] (mean)	*C. calophylla*[3] (mean)	Bee-pollen[4] (range)	Ref[5] (range/ mean)	Ref[6] (range)	Broad range Bee-pollen (estimation)	Wesson's Salt[7] (10 X)*	*Estimated requirements sufficient for honey bees*		
											Ref[8] (mg/Kg)	Ref[7] (mg/Kg)	Ref[9] (µg/bee)
Macro elements													
Calcium	1,836.	1,573.	200.–3,000.	900.	600.	82.8–305.	360.–3,100. (1,146.)	828.–4,670.	200.–5,000.	134.	1,000.	500.	12.1
Magnesium	698.	971.	200.–3,000.	500.	900.	32.1–284.	220.–2,700. (716.)	348.–3,621.	200.–4,000.	9.00	1,000.	300.	24.2
Potassium	5,543.	5,528.	4,000.–20K	4,200.	5,400.	2,353.–8,204.	2,200.–38K (5,530.)	1,431.–9,910.	2,000.–40K	41.7	5,000.	1,000.	194.
Sodium	431.	54.0	–	300.	100.	247.–846.	16.0–480. (82.2)	<0.004–1,466.	0.00–1,500.	33.0	200.	50.0	24.2
Phosphorous	–	–	800.–6,000.	3,200.	4,200.	2,136.–9,587.	1,400.–8,000. (4,600.)	2,177.–8,165.	800.–10K	–	–	–	206.
Sulfur	–	–	–	2,300.	3,000.	–	1,100.–3,700. (2,378.)	–	1,000.–4,000.	–	–	–	145.
Micro elements													
Iron	44.0	59.0	11.0–17.0	181.	124.	75.2–208.	14.0–520. (67.2)	11.1–552.	10.0–600.	2.00	–	50.0	3.63
Zinc	28.0	50.0	30.0–250.	52.0	79.0	28.25–65.3	16.0–340. (58.3)	5.10–76.1	10.0–400.	0.01	–	50.0	3.52
Manganese	13.0	37.0	20.0–110.	26.0	35.0	8.69–357.	5.00–110. (32.7)	12.0–211.	10.0–400.	0.10	–	50.0	3.63
Copper	6.00	7.00	2.00–16.0	15.0	22.0	8.31–25.1	3.00–42.0 (12.4)	3.20–25.4	2.00–50.0	0.20	–	50.0	0.56
Selenium	–	–	–	–	–	–	–	<0.01–4.45	–	–	–	–	–
Boron	–	–	–	10.0	19.0	–	–	–	–	–	–	–	–
Aluminium	–	–	–	–	–	94.5–218.	–	–	–	–	–	–	–

[1]Southern France (Rasmont *et al.*, 2005); [2]Reviewed in (Campos *et al.*, 2008); [3]Australia (Manning, 2001) In [9](Black, 2006a); [4]China (Yang *et al.*, 2013); [5]Australia (Somerville and Nicol, 2002); [6]Brazil (Morgano *et al.*, 2012); [7](Herbert and Shimanuki, 1978c) In (Atkins, Grout and Dadant & Sons, 1975); [8]Based on brood rearing ability and mean mineral bee pollen contents (Nation and Robinson, 1971b); [9]Estimations based on a meta-analysis reviewed in (Black, 2006a). *In ppm: Ca: 133,500; Mg: 9,000; K: 41,700; Na: 33,000; Fe: 2,000; Zn: 5; Mn: 100; Cu: 200 ppm.

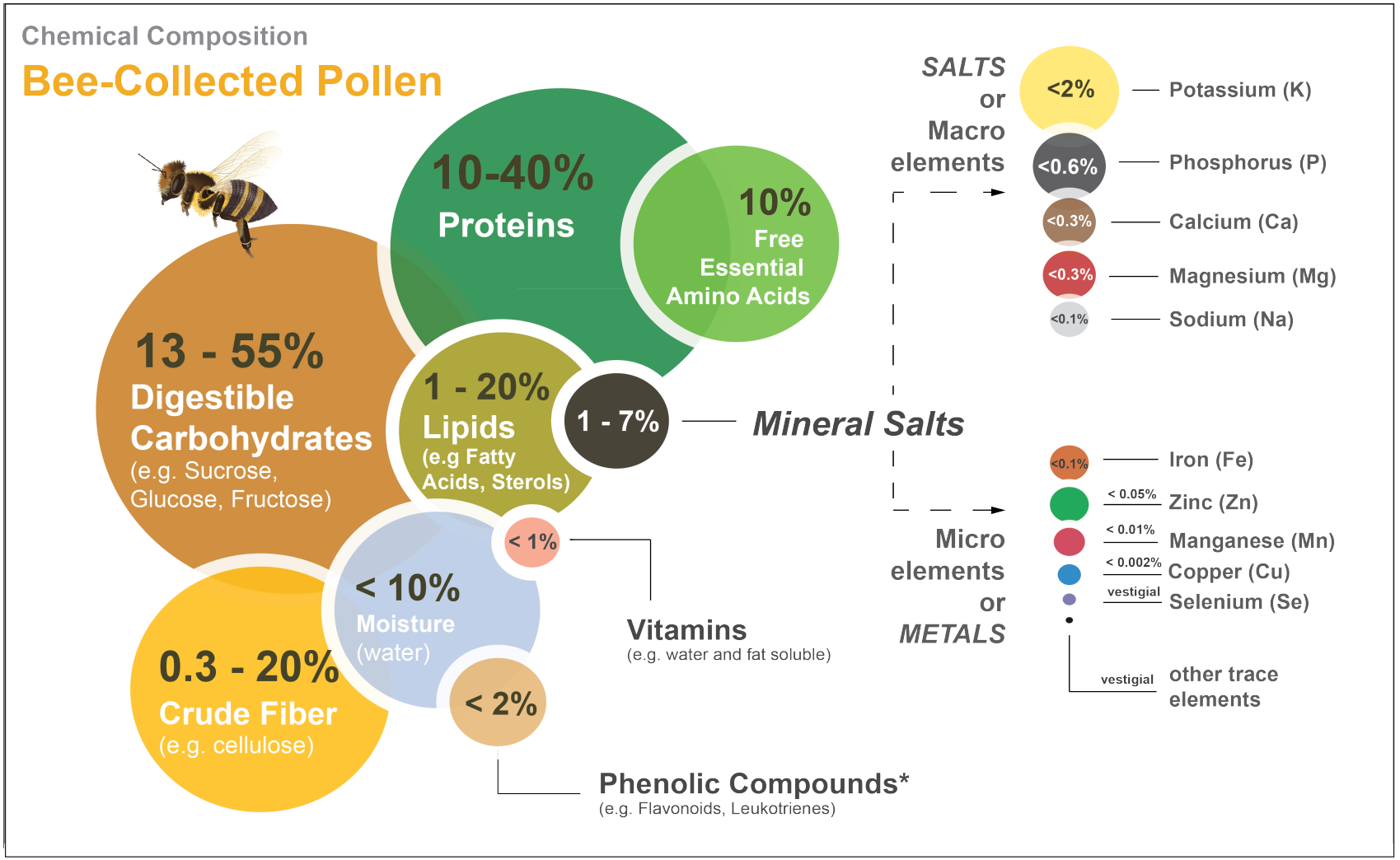

Figure 1.2 Chemical composition of bee-collected pollen based on previous literature and as displayed in Table 1.4. It is worth noting that the proportions are not at scale, but visually relative to each nutrient category. Also, despite carbohydrates make up a large portion of its chemical composition, pollen is the major source for non-carbohydrate nutrients such as proteins, amino acids, lipids, vitamins and minerals.
@ Credits to Almudena Clemente for the bee artwork.

1.6.3 Water – Source for Minerals

Water is a nutrient present in fair amounts in both body composition and most environments. To maintain osmotic homeostasis, all animals need to regulate water intake (for review see (Bourque, 2008) and adequate water supply should be acquired through diet. Most terrestrial insects are prone to great water losses and to desiccation, and thus, metabolic water may not supply all insect's water requirements, for review (Subramani and Hoek, 2010; Lowe *et al.*, 2013). Yet, few insects drink water for the solely purpose of increasing water contents (Barton-Browne, 1964); honey bees and some Lepidoptera are an exception. It is not entirely novel to our knowledge that honey bees forage on water. Water foragers can be discriminated from pollen and nectar foragers in proboscis extension response tests by evaluating their lowest sucrose response thresholds (Pankiw and Page, 2000). Within this caste, some bees forage on water, nectar or pollen, which behaviour has been associated to their individual genetics (Hunt *et al.*, 1995). Similarly, this behavioural plasticity in foraging associates with honey bee sucrose responsiveness thresholds; for review see (Scheiner, Page and Erber, 2004). Pankiw *et. al.* showed that pollen foragers demonstrate significant lower sucrose response thresholds compared to nectar foragers (Pankiw and Page, 2000). Bees collecting pollen often collect water (Pankiw and Page, 2000; Pankiw, Waddington and Page, 2001; Scheiner, Page and Erber, 2001). It is, thus, not surprising that these bees are more fine-tuned to detect minerals in food. Water foraging in honey bees occurs essentially to attain thermoregulation and feeding purposes (Lindauer, 1955; Subramani and Hoek, 2010; Stabentheiner and Kovac, 2014). In his seminal studies, Lindauer (Lindauer, 1955) reported how honey bees manage heat or cold conditions either by collecting water and promoting water evaporation inside the hive (mainly by fanning) or using water to dilute food stores and feed brood when required. In arid conditions, honey bees can collect water up to 44 mg (Visscher, Crailsheim and Sherman, 1996). Honey bees, specially nurse bees, in periods of intense brood rearing require water to produce

royal jelly from their glands to feed their young larvae (Johansson and Johansson, 1978). Nevertheless, as a highly social insect, honey bees may not require water for themselves as individuals nor experience desiccation at the extent of other terrestrial insects (Nicolson, 2009), due to their sophisticated organization as a colony. Water regulation and intake is an important source of minerals for bees, but not the main focus of this thesis.

1.7 How Do Honey Bees Taste Nutrients in Food?

Insects can perceive and learn the chemical composition of food by contact chemoreception (Marshall, 1934; von Frisch, 1934; Dethier, 1955; Whitehead and Larsen, 1976a; Whitehead, 1978). Taste is a sensory modality for evaluating the edibility of potential food sources and regulating ingestion, and, thus, taste can relate directly to an insects' nutritional needs. Unlike mammals, taste is not restricted to the mouthparts (taste buds in the tongue) (Yarmolinsky, Zuker and Ryba, 2009), but consist of hair-like cuticular extensions (taste sensilla) scattered across strategic body parts (e.g. antennae, proboscis, abdomen, tarsi). These taste sensilla are the gateway for peripheral chemical detection and the initiation of feeding in most insects (Minnich, 1932; von Frisch, 1934; Dethier, 1955; Hodgson, 1957; Whitehead and Larsen, 1976). At the cellular level, single gustatory sensilla are typically innervated by 4–5 gustatory receptor neurons (GRNs) that can respond to multiple taste qualities. These GRNs can often respond to either attractive (e.g. sugar and low salt) or aversive (e.g. bitter and high salt) chemical stimuli by inducing or suppressing feeding behaviours; for review see (Liman, Zhang and Montell, 2014) in the fruit fly or (De Brito Sanchez, 2011) in the honey bee. In contrast to olfaction and vision, gustatory responses in bees have only recently gained attention. In adult worker honey bees, gustatory chaetica sensilla are mostly found on the antennal tips, mouthparts (galea, labial palps, glossa) and forelegs (tarsi) (Marshall, 1934; Whitehead and Larsen, 1976b; Mitchell, Itagaki and Rivet, 1999) (see Figure 1.3 for an

illustration of honey bee head and proboscis). Whether bees detect specific salts by means of taste sensilla is still controversial. Recent studies suggested that honey bee tarsi are likely to be tuned to perceive salts in water (De Brito Sanchez *et al.*, 2014).

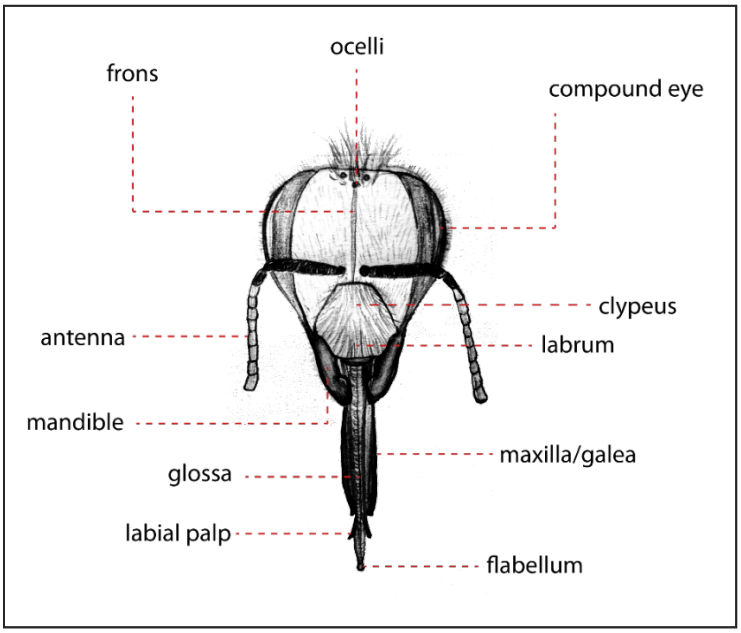

Figure 1.3 Illustration of the honey bee female head mainly composed of two compound eyes, a pair of antenna, a pair of mandibles and the proboscis, adapted from (Snodgrass, 1956). One antenna is displayed on each side of the head and is composed of 10 segments. Antennae are covered in olfatctory and taste sensillae (chaetic or basiconica) for chemoreception, with highest density on the tip. In addition to taste perception, the antennae are also reponsible for olfaction and mechanosensation. The mandibles and proboscis make up the mouthparts. The mandibles are a pair of jaws mostly used for chewing, whereas the proboscis assembles parts of the maxilla, labial palps and glossa that together produce a unique tube for lapping liquids (e.g. water, nectar). When not in use, the proboscis withdraws and folds back beneath the head. It also fuctions as a gustatory organ as it exhibits taste sensilla especially on the galea and labial palps (Whitehead and Larsen, 1976b, 1976a; Whitehead, 1978).

At the molecular level, peripheral taste in insects is mostly understood from *Drosophila* studies due to its transgenic repertoir. At least two attempts have been made into honey bee trasngenics (Schulte *et al.*, 2014; Kohno *et al.*, 2016). The

activation of GRNs within taste sensilla upon contact with dietary chemicals seems to occur via ligand-gated transmembrane proteins either directly (ion channel receptors, IRs) (Rytz, Croset and Benton, 2013; Zhang, Ni and Montell, 2013) or indirectly (G-coupled protein receptors, GRs) (Hiroi *et al.*, 2004; Montell, 2009). So far, the honey bee genome suggests a repertoire of 12 putative GRs and 21 IRs genes (Robertson and Wanner, 2006; Smith et al., 2011; Sadd et al., 2015), of which GRs appear to be expressed primarily in peripheral gustatory organs (Simcock, Wakeling, *et al.*, 2017) with three suggested as sugar receptors, AmGr1 and AmGr2 (Jung *et al.*, 2015), and AmGr3 (Takada *et al.*, 2018). Though, nothing has been yet confirmed for the remaining candidates nor for other taste modalities (e.g. salt). The appetitive pathway is tuned for low salt detection, whereas the aversive pathway prevents ingestion of high-salt food. In *Drosophila*, the cellular and molecular basis of salt taste is far better understood than in honey bees. Two epithelial Na channel (ENaC) members (*ppk11* and *ppk19*) seem to be expressed in the terminal organ and required to detect low salt in larvae (Liu et al., 2003). In adult flies, responses to low and high salt is mediated by two types of salt-responsive GRNs (Zhang, Ni and Montell, 2013). Low salt sensing in adults appears to require IR76b, from the ionotropic glutamate receptor family (Rytz, Croset and Benton, 2013). As for high salt sensing, it has been reported that two genes expressed in gustatory neurons in the terminal organ of larvae (*ppk19* and *sano*) are both required for high-salt sensing and avoidance behaviour (Alves et al., 2014). Compared to salts, the effect of metals on feeding responses is less studied, but if consumed in excess, metals can be toxic and impair health. Few studies explored the toxicity of individual metals in bees (Hladun *et al.*, 2012, 2016; Hladun, Kaftanoglu, *et al.*, 2013; Burden *et al.*, 2016). Other insects develop learned food aversions to high metals in food and internally regulate metal toxicity (Stone, Jepson and Laskowski, 2002; Behmer *et al.*, 2005; Freeman *et al.*, 2007; Grześ, 2009; Green, Diaz and Tibbett, 2010; Russell *et al.*, 2011; Hurst, Stevenson and Wright, 2014; Bednarska and Stępień, 2015; Stolpe and Muller, 2016). Yet, only a handful of reports are currently available on the gustatory responses of honey bees to dietary

metals in water or floral rewards. The effects of mineral salts on honey bee' gustatory responses and feeding behaviour needs to be further investigated.

1.8 Mineral Nutrition in Honey Bees

Like flies (Diptera) and butterflies (Lepidoptera), honey bees are holometabolous insects, i.e. larvae are different from adults, and so their nutrient needs for growth differ significantly from larva to adult (Peterson, 1964; Dadd, 1973). In addition, food sources from which insects acquire their necessary nutrients may also change. Honey bees require virtually the same type of essential nutrients as all other organisms including carbohydrates, proteins and amino acids, lipids and fatty acids, vitamins, mineral salts and water (Haydak, 1970). And as far as all nutrients are available and provided in adequate amounts and ratios, optimal nutrition is accomplished. However, honey bees live as a superorganism and, as such, the colony must deliver and respect different nutritional demands and feeding habits simultaneously. Insect societies are composed of individuals at different life stages, which impose different nutritional demands. Adequate nutrition not only should be attained by non-growing individual worker bees, either nurse or foragers, but also by developing larvae, male bees and the reproducing queen. Therefore, protein is mostly allocated to growing and reproducing individuals, whereas lipids and carbohydrates are available to all colony members as energy sources (Cassill and Tschinkel, 1999; Arganda *et al.*, 2014, 2017; Paoli, Donley, *et al.*, 2014; Stabler *et al.*, 2015; Vaudo *et al.*, 2017). Adult worker bees rely on a sugar-based diet to maintain their high demanding energetic metabolism and survive. Young adult also consume pollen for somatic maintenance, but specifically to acquire nutrients necessary to feed their developing larvae and the reproducing queen. Protein is essential for maturation of hypopharyngeal glands in nurse bees (Crailsheim *et al.*, 1992), which mediate larval nutrition by producing protein and fat-rich jelly critical for brood rearing (De Groot, 1953; Herbert and Shimanuki, 1978b, 1978c; Brodschneider and

Crailsheim, 2010). Yet, if ingested in excess and specifically in the absence of brood, significantly affects adult workers' longevity. Nonetheless, adult workers are important for colony nutrition by both producing nutrient-rich jelly (Crailsheim *et al.*, 1992; Hrassnigg and Crailsheim, 1998) and distributing the digested nutrients across nestmates (Brodschneider et al., 2017) via trophallaxis (Crailsheim, 1998) (see Figure 1.1 c). But how micronutrients are distributed among insect societies is far less understood. In honey bees, small amounts of minerals in pollen have been assumed to support development and growth (Haydak, 1970), though, not essential to support the development of hypopharyngeal glands in young nurse bees (Haydak and Dietz, 1965). Adults also require minerals throughout life to support somatic metabolism but in which extents and how mineral imbalances affect adults' health and performance remains to be formally investigated. Nutrient deficiencies are not unusual, though observable symptoms in insects are more difficult to find, especially micronutrient deficiencies (House, 1963). Micronutrients' functions (e.g. coenzymes or cofactors) are rooted to several metabolic pathways and thus, its absence may end up disrupting the production of other essential nutrients (e.g. amino acids). Like much else regarding mineral nutrition, less in known about mineral deficiencies in insects. Lack of dietary minerals or exposure to excesses have been reported for different species. For example, limitation of N, P, K or Fe impaired growth and pupation time in the butterfly *Pieris brassicae*, and limited fertility in the beetle *Phaedon cochleariae,* both reared on mineral-deficient leaves (House, 1963). Diets deficient in K, Mg or P induced empty ovaries during the yolk synthesis stage in the fruit fly *Drosophila melanogaster* (House, 1963). In the gypsy moth, dietary iron deficiency for successive generations disrupts development, growth and affects feeding responses in adults (Keena, Odell and Tanner, 1998). In bees, high levels of K, Ca, P and Na in nectar or sugar syrups provided as feed supplements can be detrimental (Standifer *et al.*, 1978; Herbert, 1979; Imdorf *et al.*, 1985). Also, high levels of Cu and Se impact negatively worker longevity and brood rearing in field colonies (Hladun *et al.*, 2016). Even though is expectable that nutrient excesses affect honey

bee health negatively (see Bertrand's rule, Figure 1.4), the extent and concentrations inducing these toxic effects or inducing deficiencies are not well characterized. This is consistent with previous recommendations to not supplementing bee food/diets with mineral salts (Williams *et al.*, 2013).

Two important studies demonstrated that systematic increase of pollen ash to synthetic diets improved brood rearing and worker lifespan, which were best at 0.5–1% added pollen ash, decreasing for levels > 2% (Nation and Robinson, 1968; Herbert and Shimanuki, 1978c). Evidence pertaining mineral requirements of honey bees is scarce and has been often inferred from the mineral composition of either bee-collected pollen or bee bodies (Manning, 2002; Somerville and Nicol, 2002; Black, 2006a; Manning, 2016). Mineral contents have been reported to increase in worker bodies up to 6 days old (Dietz, 1971). Most prevalent levels in bee bodies were found for Fe, Zn and Mn (Manning, 2002) with Mn higher in bee heads (Nation and Robinson, 1971a); for review see (Black, 2006a; Manning, 2016). Possibly Mn is important for cognition or behaviour in forager bees at appropriate levels, yet this remains to be investigated. In social insects such as ants and termites, body micronutrient levels have been proposed to match the expected nutritional requirements of different castes (Judd and Fasnacht, 2007). This study revealed that micronutrient levels in both species matched the nutritional needs between castes (growing/reproducing vs. non-growing). This indicates that mechanisms selectively regulating the intake of different elements postingestively exist. Another study has recently postulated that honey bees feeding on certain pollen types may suffer specific mineral deficiencies. This study reported limitations for S, Cu, P, K, N and Zn in some pollen types, and especially for Na, which concentrations in pollen were consistently low in relation to bee body contents (workers: ~700 ppm; queens: ~1,000 ppm) and, thus, expected requirements (Filipiak et al., 2017).

Na is a critical mineral nutrient for terrestrial animals as they constantly lose water and Na through normal physiological processes. Body fluid balance is maintained by adjusting not only water ingestion and excretion, but also Na

(Geerling and Loewy, 2008; Beyenbach, 2016). Na is often limiting for herbivores that subsist off plant tissues that often tend to be low in Na (Kaspari, Yanoviak and Dudley, 2008; Kaspari et al., 2009). Severe Na deficiency can induce death (Wilkins and Richter, 1940), therefore, animals exhibit behavioural changes associated with seeking and ingesting sodium-rich foods (Richter, 1936; Schulkin, 1991a; Hurley and Johnson, 2015). In ants, Na limitations can be counterbalanced by, for example, increased recruitment of salt baits (Kaspari, Yanoviak and Dudley, 2008; Dudley, Kaspari and Yanoviak, 2012; Hernández et al., 2012). Other studies reported cases of both solitary bees (Bänziger *et al.*, 2009; Abrol *et al.*, 2012) and social bees (Butler, 1940; Bonoan *et al.*, 2016; Lau and Nieh, 2016) foraging on 'dirty water' or other mineral-rich substrates, which is believed to function as supplementary Na intake. High salt concentrations often trigger rejection in rats (and other animals), but when they become Na deficient, these animals engage in ingesting higher concentrations of salty food (Berridge *et al.*, 1984; Bertino and Tordoff, 1988); see review (Hurley and Johnson, 2015). Honey bees, as well, tend to reject high salt (De Brito Sanchez *et al.*, 2005). However, they also need to maintain Na levels within an optimal range for proper metabolic functioning. To understand which are the behavioural and physiological acceptable levels of Na in adult honey bees, still requires further investigations.

1.9 Bertrand's rule – Eating Within the Right Range

To maintain nutritonal homeostasis (demand vs. supply) animals need to do more than just eat, they must be able to identify, evaluate, select and adjust the ingestion of specific nutrients (carbohydrates, lipids, proteins, aminoacids, vitamins, minerals). Inorganic elements were traditionally divided into toxic, innocuous or essential, depending on the health impact outcomes after ingestion (Mertz, 2009). In toxicology, a dose-response relationship is traditionally described as the change in effect induced by exposure of different levels of a drug or chemical compound.

Bertrand applied this mathematical model to nutrition by describing the potential impacts of a nutrient on health depending on the degree of exposure (by ingestion) (Bertrand, 1912). This principle supports the evidence that "essentiality does not exclude toxicity" (Mertz, 2009); and that essential nutrients can become toxic if consumed in excess. This dose-response model applied to nutrients, also termed Bertrand's rule, is depicted in Figure 1.4 It is characterized by a curve, wherein, in the context of feeding, ingestion of low concentrations of a nutrient, is beneficial (by stimulating feeding and/or fitness), whilst ingestion of high concentrations of that same nutrient (beyond the optimum) turns out detrimental (by inhibiting or decreasing feeding and fitness) (Calabrese and Baldwin, 2000). While this model was primarily believed to be true for micronutrients, now it is also believed to apply to caloric nutrients such as carbohydrates (Raubenheimer, Lee and Simpson, 2005). Whether it respects to macronutrients or micronutrients animals should demonstrate regulatory mechanisms that liaise the intake of food (and nutrients) to reach that optimum intake and an equilibrium between food components that promote the best possible fitness, while avoiding excesses. The aim of all animals is to accomplish adequate nutrition, which is to obtain all the nutrients required by the organism, within a suitable range of concentrations. Food such as this is often termed a balanced diet. In other words, a balanced diet is accomplished through the animal ability to adjust nutrient intake. Yet, ingesting food-derived nutrients from nutritionally diverse, and possibly unbalanced, foods can be challenging. Meeting nutrient requirements through feeding is not a trivial task to fulfil, and animals must be equipped with physiological mechanisms that help them to cope with nutritional challenges.

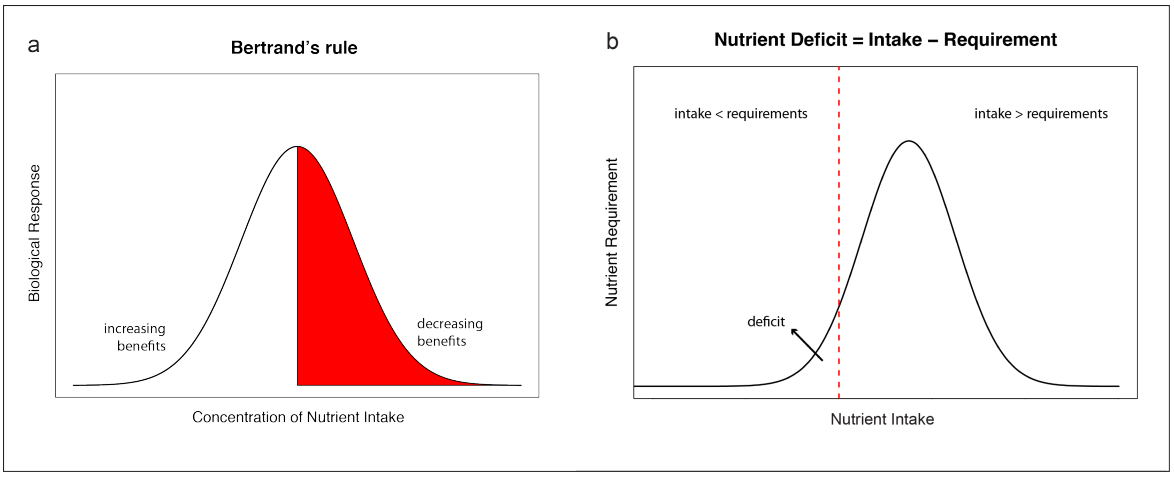

Figure 1.4 Dose-response model of nutrients adapted after (Mertz, 1981). **a)** the panel on the left depicts the general dose-response model applied to nutrient intake. This model describes the non-monotonic dose-response characterized by increasing doses associated with increased biological benefits until an optimum; at higher doses of that nutrient (nutrient intake beyond the optimal range), the probability of toxicity increases, which phenomenon is also termed hormesis (Calabrese and Baldwin, 2000); **b)** the panel on the right represents the relationship between nutrient intake and nutrient requirement. If the bulk of ingestion of a nutrient is below the set requirement (optimum), then the animal is deficient in that specific nutrient, which can also be visualized in panel a (left-blank side of Bertrand's Model). If the nutrient intake is higher than the required, then is possible that an animal ingested too much of that nutrient, and may suffer from the associated physiological consequences; adapted after (Simpson and Raubenheimer, 2012).

Balancing nutrient ingestion (nutritional regulation), whether it respects to humans or insects, drives a similar fate: reproductive success, general health and survival. Compared to macronutrients, our knowledge of insects' micronutrient requirements, whether they balance micronutrient intake and, if so, which physiological and behavioural regulatory mechanism are displayed, is still little. Nonetheless, in both mammals and insects there is evidence for regulation of salt intake around an optimal range (Stellar, 1960; Trumper and Simpson, 1993). This behavioural output of patterns of food intake across different concentrations of a salt reassembles the shape of the mathematical model of Bertrand's rule. Mineral intake must be optimised around values that promote feeding, fitness and survival. Optimisation of nutrient intake for a balanced nutrition is assisted by two main

mechanisms: behaviour (taste and preference-avoidance) and postingestive feedbacks that together regulate nutrient ingestion.

1.10 Mechanisms Regulating Salt Intake

An excellent evidence of regulation of nutrient intake and the power of gustatory inputs in main feeding decisions in insects is the "Taste Model" proposed and demonstrated by Trumper and Simpson (Trumper and Simpson, 1993; Simpson, 1994; Simpson and Raubenheimer, 2011). This model proposes that food items diverge in palatability (phagostimulatory power) across concentrations of salt, and advanced that the optimal concentration elicits the "best taste" or phagostimulatory power. The preferred diet is thus the one more attractive by taste. The phagostimulatory power was taken as the measure of the likelihood to initiate feeding. Fifth instar locusts were offered a choice between two complete foods differing in salt (mixture) content but including the preferred optimum salt concentration able to independently regulate the intake of salt and macronutrients to the optimum intake. However, when locusts were restricted to a single food, these animals regulated the intake of the non-mineral components, no matter how much (deficit or excess) of salt was ingested. This study showed for the first time, that insects can regulate the salt intake in relation to macronutrient composition if freely able to choose and if nutrient concentration covers the optimum concentration. The same study emphasized that salt regulation could be achieved due to the phagostimulatory power of the food (taste attractiveness) equals the required amounts of salt needed to reach the optimal target (around 1.8% of Wesson's salt mixture in food containing 20% proteins and 10% digestible carbohydrates). The best it tastes, the greater the phagostimulatory power and the increased likelihood of food acceptance. If we recall the shape of Bertrand's rule, animals should first self-select a food source that provides the optimum range of nutrients (e.g. salt); if there is no optimal food available, but rather food with nutrient contents above and below the

preferred optimum, animals may still be able to adjust nutrient intake and satisfy their nutritional needs. By gathering and reporting behavioural and physiological data, Trumper and Simpson postulated that optimisation of salt intake is accomplished through mechanisms involving increased locomotion, gustation (phagostimulatory power/food palatability cues), postingestive feedbacks (e.g. haemolymph osmolality feedback) and associative or non-associative learning (e.g. neophilia) to accept or reject food (Trumper and Simpson, 1993, 1994). Similar evidence has been observed in rats. Rats presented with a choice of water or salt (NaCl) solutions, they drank increasing concentrations of saline until a preferred (optimal) concentration was reached and, beyond which, ingestion decreased (Stellar, 1960; Schulkin, 1991b; Simpson and Raubenheimer, 1996).

In the context of Na deficiency, many animals across taxa engage in a motivated behavioural state that drives them to seek and ingest foods that contain Na; for review refer to (Schulkin, 1991a, 1991b; Hurley and Johnson, 2015). This behaviour, also termed salt/sodium appetite, is an innate regulatory mechanism that ultimately directs animals to seek, detect and ingest specific foods to restore Na levels. Seminal works conducted by Curt Richter (Richter, 1936) in rats demonstrated that animals unable to retain Na and, thus, rendered Na-deficient will voluntarily ingest elevated levels of saline to survive. This change in behaviour (acceptance of high concentrations and overingestion of saline) occurred in response to Na deficiency. Saline intake was restored to normal levels when adrenalectomised rats were transplanted functional adrenal tissue and the production of aldosterone hormone (for urinary Na conservation) was re-established (Richter and Eckert, 1938). It is not likely that honey bees ever face such dramatic salt deprivation contexts. However, pollen composition is diverse and not all pollen types present the same mineral contents, which also tend to change with season (Bonoan *et al.*, 2016). As previously mentioned, some insect species have already been observed engaging in behaviours that are believed to be a form of supplementary feeding targeted at specific micronutrients – puddling behaviour; for review refer to (Molleman, 2010). Among

insects reported to feed on excrements, 'dirty water', urine and secretions of vertebrates such as sweat and tears are honey bees (Apidae) (Butler, 1940), sweat bees (Halictidae) and stingless bees (Apidae) (Bänziger *et al.*, 2009), and locusts (Shen *et al.*, 2009). A lack of protein and salt may further drive more extreme behaviours such as cannibalism in crickets (Simpson *et al.*, 2006), and possibly in honey bees (Schmickl and Crailsheim, 2001). Altogether, and depending on the context of the animal, these behavioural and physiological mechanisms can assist regulation of mineral intake.

1.11 Behavioural Regulation of Nutrient Intake in a Superorganism

Most animals eat to satisfy their nutritional needs and survive, in contrast to humans that are occasionally motivated to eat driven by hedonic hunger. In any case, dietary self-selection is pervasive among animals (Waldbauer and Friedman, 1991). Self-selection assumes that food intake is not random; similar individuals tend to select nutrients in the same proportions; and individuals able to self-select perform better in the absence of optimal food sources (Waldbauer and Friedman, 1991). As previously mentioned with regards to the "Taste Model", locust nymphs restricted to diets varying in salt concentrations were not able to regulate salt intake (Trumper and Simpson, 1993). However, when given a choice, behavioural regulation of salt intake was possible though adjusting consumption between low and high salt mixture contents (Trumper and Simpson, 1993, 1994), and rejection of toxic high salt diets (Bernays and Lee, 1988). To my knowledge, this was the first study elucidating behavioural optimisation of salt intake in insects. However, that study used a series of dilutions from a standard salt mixture (Wesson's Salt Mix) used for vertebrate livestock, which proportions between minerals may not be the most adequate for insects (Cohen, 2015). The role of dietary NaCl in a concentration gradient on preference-rejection behaviours has been studied in fruit flies (larvae and adults) (Niewalda et al., 2008; Russell et al., 2011), kissing bugs (Pontes, Pereira and Barrozo,

2017), butterflies (Inoue et al., 2012) and rats (Contreras and Kosten, 1983). Therefore, minerals in food can influence food acceptability by working either as phagostimulants, activating gustatory pathways that respond to different salts and stimulating feeding (positive stimulus) (Dethier, 1976; Trumper and Simpson, 1993); or as phagodeterrents that inhibit feeding (negative stimulus) (Simpson and Raubenheimer, 2000; *Insect Taste, Volume 63*, 2008). Salt alone (NaCl) is avoided by locusts (Chapman, 1988), but mixed in food enhances flavour (Breslin and Beauchamp, 1996). Also, feeding on free or low salt diets may trigger a build-up of other nutrients in the hemolymph and influence postprandial regulatory mechanisms and ultimately inhibit feeding (Simpson and Raubenheimer, 1993; Trumper and Simpson, 1994). Previous experience of the animal exposed to a food source can also influence its behaviour towards that same food in a second exposure. Associating a stimulus with a reward (e.g. increased fertility it growth) or with a punishment (e.g. postingestive-induced toxicity) can also influence food intake (Simpson and White, 1990; Wright *et al.*, 2010; Wright, 2011). Ingestion behaviours of metal nutrients are far less studied, though, dietary preferences for Fe, Cu and Zn were demonstrated in larvae and adult fruit flies (Bahadorani and Hilliker, 2009); for a review see also (Mogren and Trumble, 2010).

Nutrient imbalances are common because access to food is not always possible, especially to chemically balanced diet containing adequate proportions of nutrients (House, 1969); see also (Simpson and Raubenheimer, 2012). The relationship between deficiency-toxicity of micronutrients follows a dose-response model, termed– Bertrand's rule. As above mentioned (section 1.9; refer to Figure 1.4) an increase in health benefits occurs with the ingestion of low levels of a nutrient until an optimum threshold; further ingestion translates in increased costs as the regulatory mechanisms become overwhelmed and excesses become toxic and potentially lethal (Bertrand, 1912; Raubenheimer, Lee and Simpson, 2005). Optimisation of nutrient intake for adequate nutrition can, therefore, be attained through mechanisms regulating nutrient intake. A wide range of animals can achieve nutritional

homeostasis through behavioural regulation of food intake, involving the selection and preference-aversion behaviours and/or postingestive feedbacks such as physiological adjustments of the rate of excretion, for review see (Behmer, 2009; Simpson and Raubenheimer, 2012). Like solitary insects, increasing evidence suggests that social insects are, as well, macronutrient balancing organisms. Regulation of nutrient intake in a superorganism can work such as foraging insects act as a proxy for preingestive pathway by assessing nutrient quality of available foods and collecting it for the colony; and larvae could function as "internal nutrient sensors" that provide feedback on the nutritional state and needs of the colony (postingestive mechanisms) (Dussutour and Simpson, 2009). For example, at the individual level, adult individuals mostly rely on the ingestion of carbohydrates to fuel their metabolic needs, while the major sink of proteins is developing larvae in species of both bees and ants (Dussutour and Simpson, 2008, 2009, 2012; Altaye *et al.*, 2010; Paoli, Donley, *et al.*, 2014; Stabler *et al.*, 2015). Bees and ants cannot only regulate the intake of macronutrients to optimise fitness traits against high nutrient variation, but also, balance the ingestion of multiple nutrients according to colony needs (Cassill and Tschinkel, 1999; Dussutour and Simpson, 2008, 2009; Altaye *et al.*, 2010; Cook and Behmer, 2010; Cook *et al.*, 2010, 2012; Pirk *et al.*, 2010; Paoli, Donley, *et al.*, 2014; Stabler *et al.*, 2015; Vaudo *et al.*, 2016, 2017; Hendriksma and Shafir, 2016; Zarchin *et al.*, 2017). Regulation of protein intake is important as overingestion of nitrogen-rich diets above the required ratio and amount increases adult worker mortality and shortens lifespan in social insects (Dussutour and Simpson, 2012; Paoli, Donley, *et al.*, 2014).

Protein-rich pollen has been reported to be preferentially collected by bumblebees (Leonhardt and Blüthgen, 2012; Konzmann and Lunau, 2014; Muth, Francis and Leonard, 2016), though regulation of protein intake may not necessarily occur through preingestive mechanisms such as taste in honey bees (Pernal and Currie, 2002; Roulston and Cane, 2002; Cook *et al.*, 2003). These works together imply that, in contrast to bumblebees, individual honey bee foragers may not be able to

discriminate pollen quality based on protein or other nutrients content, i.e. via gustatory assessment. As in the case of nectars, minerals in pollen seem as well to improve palatability (given the right concentrations). This appears to be consistent to the fact that, in contrast to pollen proteins, minerals are unlikely to require digestion to become bioavailable to bees. As such, both pollen foragers and nurse bees are likely to assess pollen mineral composition via preingestive pathways. Whether bees perceive minerals in pollen, distinguish between different minerals and optimise its intake is not known. In turn, minerals in royal jelly seem to range between 0.8% and 3% (Sabatini et al., 2009), but most importantly its mineral composition remains fairly constant (Stocker et al., 2005; Wang et al., 2016; Balkanska, Mladenova and Karadjova, 2017). Together, these authors reported higher levels of Zn, Cu and Fe, but similar Mn levels compared to honey and pollen (percursors of jelly production). This suggests that homeostatic mechanisms, operating possibly at the level of hypopharyngeal glands, can buffer dietary mineral variation and maintain an optimal range of minerals in larval food (Stocker et al., 2005; Wang et al., 2016; Balkanska, Mladenova and Karadjova, 2017). Two other studies showed that a systematic increase of pollen ash in synthetic diets favoured brood rearing and worker lifespan, which were best at 0.5 –1% pollen ash, decreasing for levels > 2 % (Nation and Robinson, 1968; Herbert and Shimanuki, 1978c). From these previous studies, the optimal range of mineral salts in bee diet, both bee-collected pollen and glandular jelly, is consistent (0.5–3%). Evidence for micronutrient (vitamins, mineral salts) regulation at the collective and individual level in bees is still absent. Recently, termites (Blattodea: *Reticulitermes flavipe*), a social insect with different colony structure, were reported to balance their intake of mineral nutrients by adjusting consumption rates between two complimentary foods of KCl, $MgSO_4$, and $FePO_4$ (Judd *et al.*, 2017). These insects consistently overconsumed KCl or $FePO_4$ to prevent consuming too much of $MgSO_4$. Taken together, it is possible that adult insects too require minerals for a multitude of purposes besides growth. But, whether adult bees

perceive minerals in pollen, distinguish between different minerals and optimise its intake is not well understood.

1.12 Significance of this Study

Why do we need to have a better understand better adult workers feeding strategies and which are their feeding preferences? Previous studies estimated that 70% of main crops used for human consumption worldwide (Klein *et al.*, 2007) and better quality crops (Klatt *et al.*, 2014) require pollinators. Moreover, Gallai et al. estimated the economic value of pollinators around € 153 billions (9.5 % total value of food production) (Gallai *et al.*, 2009) whereby honey bees are of foremost importance (Watanabe, 1994; Breeze *et al.*, 2014). Therefore, honey bees are one of the most common and the leading managed insect pollinator worldwide (Klein *et al.*, 2007; Potts *et al.*, 2016). Honey bees may well rank third as the most valuable managed livestock in Europe (Tautz, 2008). Crop pollination is a livelihood and a by-product of honey bee foraging behaviour; for plants, it is the propagation of life; and for the honey bees, it is their way of accomplishing nutrition for the whole colony. Yet, critical drivers compromising bee health are land use and monocultures, pesticide use, climate change, hive management and pathogens (Goulson *et al.*, 2015; Potts *et al.*, 2016). Pollinators' malnutrition is one of the most critical factors that should be taken into consideration. Nutrients in pollen, but also nectar (Nicolson, 2011), can influence: bee immunocompetence (Mao, Schuler and Berenbaum, 2013) by boosting detoxification pathways; development and brood rearing (Crailsheim *et al.*, 1992; Brodschneider and Crailsheim, 2010); food preferences and behaviour (Wright, 2011; Wright *et al.*, 2013; Nicholls and Hempel de Ibarra, 2016) by modulating recognition of food rewards and foraging decisions; and lifespan (Paoli, Wakeling, *et al.*, 2014; Wang *et al.*, 2014). Good quality nutrition assumes an optimal proportion of all the essential nutrients (proteins, amino acids, lipids, vitamins and minerals). However, nutritionally-complete floral resources are difficult to find in nature due to changing

landscapes (Simpson and Raubenheimer, 2011), especially regarding mineral contents (Herbert and Shimanuki, 1978a; Morgano *et al.*, 2012; Donkersley *et al.*, 2014). Besides, intense agriculture can deliver a monotonous diet source by decreasing diversity of floral resources and negatively impact the health and fitness of bee colonies (Somerville, 2001; Di Pasquale *et al.*, 2013, 2016). In addition, changes in pollen quality and pollen shortages have been indicated as major factors that can affect bee health by increasing the nutritional stress (Arien *et al.*, 2015; Frias, Barbosa and Lourenço, 2016) and the ability to cope with toxin break down and pathogen infections (Hayden, 2000; Brodschneider and Crailsheim, 2010; Alaux *et al.*, 2011). Most of these effects have been mainly attributed to macronutrients present in pollen. The nutritional importance of micronutrients (e.g. salts and metals), its requirements, deficiencies and toxicity are well-established for animal vertebrates, especially for humans (FAO *et al.*, 1998) and livestock (cattle, pork and poultry) (Hidiroglou, 1982). In a recent meta-study, Filipiak et al. highlighted the importance of inorganic nutrient proportions in pollen to ensure a chemically balanced diet for honey bees. By comparing elemental composition of bee-collected pollen types and adult bee bodies varying in caste, they proposed that feeding on nutritionally unbalanced pollen types can result in a stoichiometric mismatch (greatest for Na) between micronutrient needs and availability (Filipiak et al., 2017). These nutritional limitations can impose constraints on bee growth and development, and overall colony health (Di Pasquale *et al.*, 2013, 2016).

We still know very little about adult insects' mineral requirements, feeding acceptance-rejection thresholds, intake regulation, and impacts of deficiencies/excesses on health (House, 1963; Haydak, 1970; Dadd, 1973). This importance is greater for insect societies wherein nutritional requirements vary greatly between colony individuals (Cassill and Tschinkel, 1999; Behmer, 2009; Cook *et al.*, 2012; Paoli, Donley, *et al.*, 2014). Therefore, elucidating the roles and impacts of understudied elements in adult worker bee nutrition is necessary. Studies, such as

this, are certain to add missing components to ultimately unveil the overall picture of honey bee feeding strategies and adequate nutrition.

1.13 Study Goals and Specific Aims

This study was motivated by the lack of information on mineral salt taste, feeding preferences and regulation in adult honey bees. Also, my goal was to draw attention to these less considered aspects of honey bee feeding behaviour and nutrition. Investigating how insects detect and respond to chemicals in food, and how they regulate its intake is important. This knowledge can foster our understanding on, for example, plant-pollinator interactions or alternative pest control techniques. But most importantly, and in the current study, increases our understanding on adult bees' mineral requirements and behaviour. In the light of this, the specific aims of this research project were:

1) To assess forager honey bees' innate sensitivity and detection thresholds to mineral salts in two dietary contexts (water or nectar-like solutions) – Chapter 3;

2) Test behavioural gustatory responses to mineral solutions in two gustatory organs involved in assessing food quality (antenna or proboscis) – Chapter 3;

3) Ascertain whether all mineral salts elicit similar gustatory responses – Chapter 3;

4) Test how sucrose gradient affects gustatory perception of salt – Chapter 3;

5) Determine a range of concentrations for each mineral tested that supports feeding and survival of young bees – Chapter 2 and 4;

6) Determine the effect of dietary minerals in the feeding responses (consumption) and self-selection behaviour of young worker bees in two-choice feeding assays – Chapter 4;

7) Determine whether the Bertrand's rule predicts optimal mineral intake and behavioural regulation – Chapter 4;
8) Ascertain whether adult workers regulate their intake of mineral salts (preingestively and/or postingestively) – Chapter 4.

1.14 Experimental Approach

To assess gustatory responsiveness and feeding preferences of adult worker bees to a range of liquid diets spanning different types and concentrations of mineral salts, two main experimental approaches were employed: **Behavioural Gustatory Assays** (Classical Proboscis Extension Reflex, PER) using harnessed forager bees in Chapter 3 (Aims 1–4); 2), and **artificial two-choice feeding assays** using cohorts of free-flying newly emerged bees in Chapter 4 (Aims 5–8).

1.15 Roadmap and Structure of this Thesis

This thesis comprises five Chapters: **Chapter 1**, General Introduction and Goals; **Chapter 2**, Optimisation of Feeding Assays; **Chapter 3**, Behavioural Gustatory Assays using forager honey bees; **Chapter 4**, Two-Choice Feeding Assays using Cohorts of Young Workers; and **Chapter 5**, Concluding Remarks and Outlook. Additionally, at the end of this document is displayed one Appendix (A) that provides supplementary information to backckup experimental designs and results. The next **Chapter 2** will address a series of pilot experiments required to optimise data collection and processing of feeding assays further described in Chapter 4. It comprises five preliminary studies: range-finding for consumption and survival support, and testing experimental box designs (Study 1); measuring the impact of incubators' shelf position on evaporation loss from water tubs (Study 2); testing methodologies to correct consumption for evaporation loss from liquid diet solutions (Study 3); testing diet delivery regimes (feeding tubes' position) on the magnitude of

food consumption (Study 4); and testing the number of bees per feeding box on the reliability of consumption measurements (Study 5). Chapter 2 is divided in eight main sections: Abstract, background, Study 1, 2, 3, 4 and 5, and Conclusion. Each Study (hypothesis testing) is then divided into three sub-sections: Rationale & Methods, Results and Discussion. In **Chapter 3**, I will address the rationale and results from the gustatory responses (antennae/proboscis) of forager bees to single minerals in either water or nectar-like solution (1.0 M sucrose). **Chapter 4** will cover the rationale and results obtained from feeding responses (consumption), preferences and survival of newly-emerged bees to single mineral diets. Both experimental Chapters 3 and 4 exhibit seven main sections: Highlights, Abstract, Background, Methods, Results, Discussion and Conclusion. **Chapter 5** will summarise the work documented throghout and emphasize the biological significance of this study and possible implications in other (practical) area.

2

Optimisation of Feeding Assays: Mineral Range Finding Experimental Protocol and Data Processing

Chapter 2

Optimisation of Feeding Assays: Mineral Salt Ranges, Experimental Protocol and Data Processing

2.1 Highlights

- Confirmation of a suitable range that supports survival for each mineral salt feeding treatment was found. Acrylic boxes are easier to manipulate and to reproduce results (Study 1).
- Shelf position inside incubators maintaining worker bee experimental cohorts, influences the rate of solution evaporation (Study 2).
- Different methodologies to correct for evaporation losses affect the magnitude of consumption measurements (Study 3).
- Diet tubes' position within the feeding box affects the magnitude of consumption measurements (Study 4).
- The number of worker bees per feeding box, specifically in high mortality-induce treatments, affects the reliability of consumption measurements (Study 5).

2.2 Abstract

A diversity of studies have used caged honey bees to study nutrient regulation under *in vitro* conditions. For example, Paoli et al. investigated how worker honey bees varying in age regulate the intake of different ratios of essential amino acids to carbohydrates (Paoli *et al.*, 2014). More recently, Brodschneider et al. examined food consumption and trophallaxis between caged honey bee cohorts varying in number and age of individuals (Brodschneider *et al.*, 2017). Although, effective standard guidelines for maintaining adult worker honey bees in caged cohorts have been reviewed (Human *et al.*, 2013; Williams *et al.*, 2013), cage designs vary greatly among research groups. This Chapter encompasses the pilot experiments I found necessary to study bee mineral salt preferences and intake regulation under *in vitro* conditions prior scalling up main experiments. Therefore, here, I address five preliminary studies: range-finding for consumption and survival support, and testing experimental box design (Study 1); measuring the impact of incubators' shelf position on evaporation loss from water tubs used as a proxy for liquid diet evaporation (Study 2); testing methodologies to correct consumption for evaporation loss of liquid diet solutions (Study 3); testing diet delivery regime (feeding tubes' position) on the magnitude of food consumption (Study 4); and testing the number of bees per feeding box on the reliability of consumption measurements (Study 5). After this abstract, this Chapter is divided in seven main sections: Background, Studies 1, 2, 3, 4 and 5, and Conclusion. Each Study section (hypothesis testing) is then divided into Methods and Rationale, Results and Discussion. These data together helped finding a working range of sublethal concentrations for individual mineral salts, but also to an improved experimental design and methodolgies to conduct feeding assays using adult worker bees later in Chapter 4.

2.3 Background

European honey bees are a well-known managed pollinators that are important for the world economy and our food production systems (Klein *et al.*, 2007; Aizen *et al.*, 2009; Gallai *et al.*, 2009; Potts *et al.*, 2016). Declines in bee populations have been extensively reported and several factors such as poor nutrition have been proposed as threatning agents encompassing these losses (VanEngelsdorp *et al.*, 2011; Vanbergen and Garratt, 2013; Goulson *et al.*, 2015; Desmedt *et al.*, 2016; Hallmann *et al.*, 2017). As such, studying honey bees' feeding behaviour across different aspects of the nutritional spectrum is important. For example, studying honey bees under laboratory conditions is an effective first approach to understand how different food regimes and diets affect health and performance of whole colonies. These may later translate into large-scale field experiments, and provide more comprehensive evidence on factors influencing bee fitness.

Most of our basic current knowledge on honey bee qualitative nutrition is derived from landmark studies conducted during the 1930s-1970s (for a review see (Haydak, 1970; Brodschneider and Crailsheim, 2010). More recently, quantitative nutrition and nutritional regulation have gained attention and can deliver novel insights into the intricate dynamics of honey bee individuals and social nutrition. These studies often consist of measuring at least two key variables in laboratory conditions: food consumption and bee survival. To study food selection, the most common protocols for measuring feeding regulation are 'two-choice' and 'no choice' assays. **Choice assays** permit the measurement of a preferred 'intake target' attained through dietary self-selection. **No-choice assays** can be used to reveal how feeding on a restricted diet affects overall consumption and survival (physiological/behavioural outputs of feeding on imbalanced diets, i.e. above or below the self-selected or "optimum"diet).

Several studies have recently investigated how honey bees regulate the intake of nutrients and how these different food regimes affect development, growth, preferences, performance and survival (Altaye *et al.*, 2010; Pirk *et al.*, 2010; Archer *et*

al., 2014; Paoli *et al.*, 2014; Démares *et al.*, 2016; Helm *et al.*, 2017). While this is mostly the case for macronutrient regulation (e.g. proteins, free amino acids, carbohydrates), micronutrient regulation, i.e. salt intake, remains unknown in honey bees. Mineral salts are important for several metabolic reactions and sustain water homeostasis. Pollen consumption, the main source of non-carbohydrate nutrients for bees, is thought to be the main way that bees obtain the trace amount requirements (Haydak, 1970), though nectar also contains minerals and other micronutrients (Nicolson, 2011; Afik *et al.*, 2014). Previous studies proposed that mineral requirements for optimum brood rearing and worker survival must be below 3% of pollen ash (Nation and Robinson, 1971; Herbert and Shimanuki, 1978). Since then, few studies have addressed the mineral nutrition of adult honey bees.

My aim in this Chapter was to find a range of concentrations that sustain worker bee survival for eight individual mineral salts. This Chapter represents the pilot experiments I performed to study bee mineral salt preferences and intake regulation under *in vitro* conditions in greater detail in the following Chapters. In addition, I also present data for the optimisation of the experimental protocols used. A diversity of studies have used caged honey bees to study nutrient regulation under *in vitro* conditions. For example, Paoli et al. investigated how worker honey bees varying in age regulate the intake of different ratios of essential amino acid to carbohydrates (Paoli *et al.*, 2014). More recently, Brodschneider et al. examined food consumption and trophallaxis between caged honey bee cohorts varying in number and age of individuals (Brodschneider *et al.*, 2017). Although, effective standard guidelines for maintaining adult worker honey bees in caged cohorts have been reviewed (Human *et al.*, 2013; Williams *et al.*, 2013), cage designs vary greatly among research groups. Cage or hoarding box designs can vary in building material (e.g. acrylic, plastic, wood, stainless-steel), shape and size, but also in the type of feeder used (e.g. syringe, centrifuge tubes, plastic pippettes, glass tubes or bottles). This situation arises to meet individual research needs, but could lead to barely comparable results. Three independent studies have analysed these differences and proposed cage designs producing low pathogen load, leak-proof feeders and good

bee survival (Evans *et al.*, 2009; Williams *et al.*, 2013; Huang *et al.*, 2014). These studies showed that varying cage features affected measured variables (e.g. leak and drip-proof feeders, contamination) and honey bee health (e.g. pathogen load, ventilation, steady food supply). When food solutions are used in nutritional experiments with bees, consumption measurements from liquid diet solutions are often corrected by simple subtraction of mass loss measured in mock evaporation boxes (Williams *et al.*, 2013). Yet, nuances such as how different methodologies accounting for evaporation losses affect the magnitude of consumption measurements is often overlooked and could have a profound impact on the measurement of the amount of food consumed. This Chapter addresses five preliminary studies: concentration range-finding for consumption and survival support, and testing experimental box design (Study 1); measuring the impact of incubators' shelf position on evaporation loss from water tubs (Study 2); testing methodologies to correct consumption for evaporation loss of liquid diet solutions (Study 3); testing diet delivery regime (feeding tubes' position) on the magnitude of food consumption (Study 4); and testing the number of bees per feeding box on the reliability of consumption measurements (Study 5). Data and knowledge gathered throughout this Chapter informed better how to conduct further feeding experiments later described in Chapter 4.

2.4 Study 1. Find a Concentration Range That Supports Survival (Part I) and Test the Experimental Box Design (Part II)

2.4.1 Study 1. Rationale & Methods

Here, I used no-choice feeding assays to assess whether adult honey bees would ingest sucrose solutions laced with one of eight salt/metals at increasing concentrations (Figure 2.1), and its effects on bee survival over 7 consecutive days – Part I. I also tested which of the two box desings availabe in our lab provided the most reliable handling and measurements (Figure 2.2) – Part II. Briefly, newly emerged bees were collected from suitable sealed brood frames kept inside incubator

chambers 2–3 days prior eclosion (see Figure 4.1, Chapter 4). Brood frames were selected from up to five colonies kept in our apiares (Buckfast stocks). Emerging bees were brushed of the frames, counted and randomy assigned to experimental boxes in groups of 50–96 bees/box. Diet treatments consisted of sucorse (1.0 M) solutions laced with a single salt/metal at three levels of concentration (low, medium and high). Eight of the most abundant minerals in bee pollen were chosen: sodium (NaCl), potassium (KCl), calcium ($CaCl_2$), magnesium ($MgCl_2$), iron (as citrate), copper ($CuSO_4$), zinc ($ZnCl_2$) and manganese ($MnCl_2$). For reagent details refer to S1 Table, Appendix A. Concentrations were chosen according to the average mineral composition of bee-collected pollen (refer to Table 1.3 and 1.4, Chapter 1), following recommendations by (Herbert and Shimanuki, 1978).

Experimental design: Control feeding treatments consisted of sucrose only solutions. Each treatment included N= 2–3 boxes with two feeding tubes per diet (~5 mL) and one tube of distilled water. Feeding tubes consisted of modified 2 mL centrifuge tubes with 3–4 holes drilled lengthwise (Ø 2 mm) to allow lapping. To control for solution loss by evaporation, a mock evaporation box (i.e. same set up, but no bees, N= 1) was used per treatment. Consumption was measured by recording the weight difference of diet tubes every day (18–26 h). The final figure for total consumption per box per diet was adjusted for evaporation loss by subtracting the diet solution loss from mock evaporation boxes. To assess the impact of diet treatments on adult bee survival, the number of dead bees was counted daily. Dead bees were removed from each box daily.

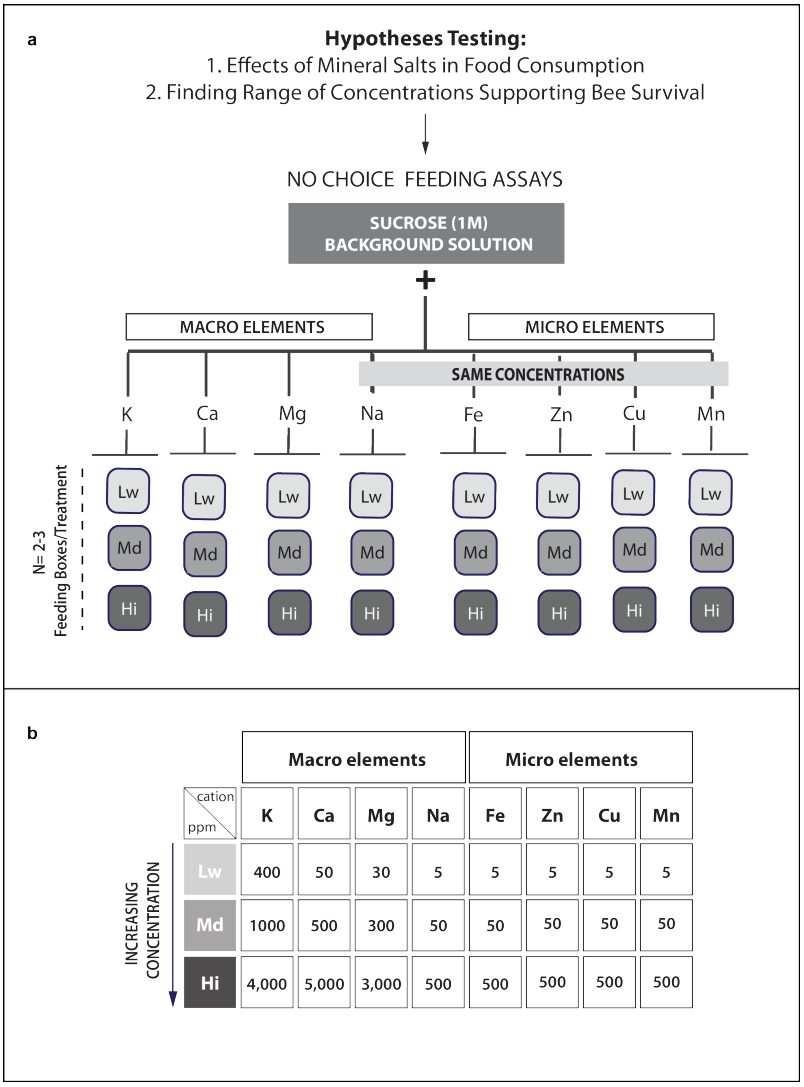

Figure 2.1 No choice assays and diet treatments. **a) pilot research design of experiments**. Newly emerged bees were restricted to sucrose solutions (1.0 M) enriched with one of eight salts/metals at three levels of concentration (low (Lw), medium (Md) and high (Hi). Four salts (macroelements: Potassium (K), Calcium (Ca), Magnesium (Mg), Sodium (Na) and four metals (microelements: Iron (Fe), Zinc (Zn), Copper (Cu), Manganese (Mn)) were tested. Boxes were the experimental unit, each treatment having N= 2–3 boxes with ~50–96 bees. Treatments were measured independently and daily over 7 consecutive days. Dead bees were counted daily. **b) Levels of concentration for each mineral salt in ppm units**. One single treatment consisted of one level of mineral salt, ranging from low (Lw), medium (Md) and high (Hi). Control treatment consisted of sucrose only solution. Distilled water was also provided *ad libitum*. Concentrations were based onTable 1.4 (Chapter 1), which reports the mean mineral pollen contents. Medium (Md) concentrations for each salt/metal was considered the optimal concentration by (Herbert and Shimanuki, 1978).

Two types of box design were used here to test whether one set up would produce better handling and measurements than the other. Twenty five treatemnts were conducted, and for a whole treatment I used one of the model designs (e.g. acrylic box). For instance, another treatment would be composed of the second model design (plastic boxes). Single boxes were considered feeding unit replicates. Figure 2.2 depicts the two box model designs: customised-manufacture vs. standard-manufacture. The first model is made of transparent acrylic (external dimensions: 13 x 11 x 4 cm; 0.4 L capacity) (Bay Plastics Ltd., UK) and exhibits two sliding screens, ventilation holes (Ø 2 mm) and six entries (3 on each side) to fit feeding tubes (modified 2.0 mL centrifuge tubes, Ø 10.8 mm). The second model is commercially available (Really Useful Boxes®) and made of semi-transparent polypropylene plastic (external dimensions: 19.5 x 13.5 x 11.0 cm; 1.6 L capacity). These standardised boxes display a pair of shallow handles on either side enabling a lockable lid, and raised edging around the lid for secured stacking of other boxes. These boxes were adapted for our purposes by drilling ventilation (Ø 2 mm) holes scattered on the top and both lateral sides. Openings (Ø 10.9 mm) to insert feeding tubes were also drilled onto the front and back sides (2 or 3 holes per side). Qualitative and informal assessments between these two models will be based in three main parameters: manipulation and material (e.g. preparation, stacking, hygiene), ventilation and tube delivery, and reproducibility of measurements.

Box treatments and controls were kept at 34 ºC in the dark inside ventilated incubators (Sanyo MIR-553) (see Figure 2.3) with four water tubs (one/shelf) to maintain air humidity. Laboratory conditions and guidelines to maitain adult honey bees in cage laboratory cohorts were followed after (Williams *et al.*, 2013). Consumption data analysis was qualitative and only suggestive of overall consumption as statistical analaysis was not possible due to low sample size (N=2–3 boxes/treatment). Survival analaysis was performed using the nonparametric Kaplan-Meier (KM) method to assess differences between survival curves across treatments (GraphPad Software, Inc., Prism 5 for Mac OS X, version 5.0a, 2007).

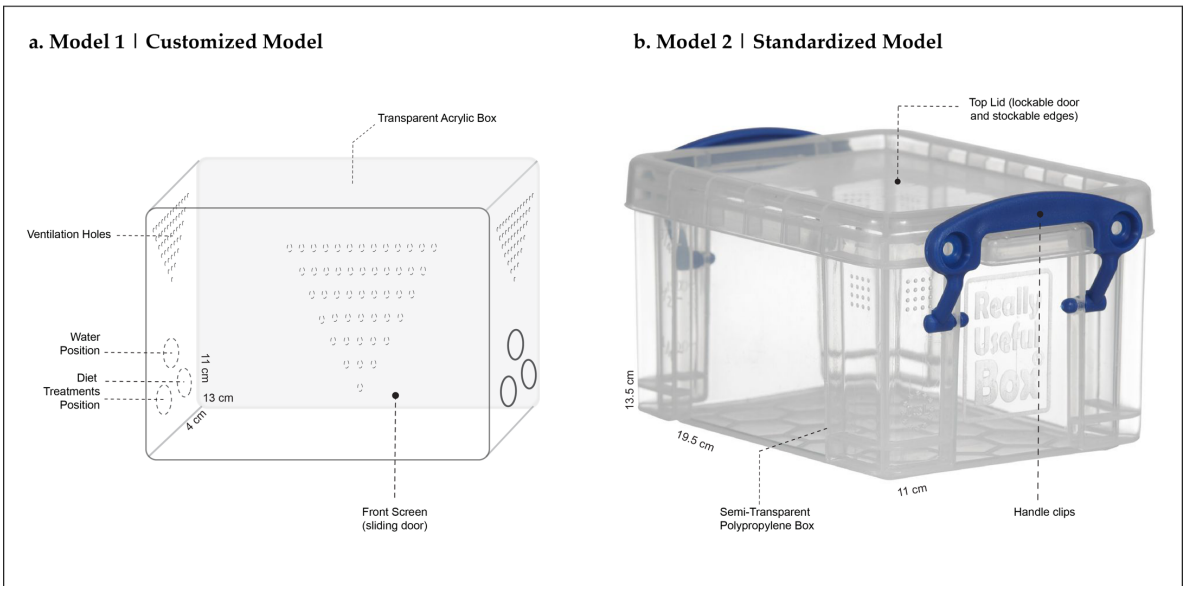

Figure 2.2 Experimental box units tested in preliminary feeding assays. Two models were tested to assess food intake in *in vitro* adult honey bee cohorts. a) mechanically customised acrylic box (external dimensions: 13 x 11 x 4 cm; 0.4 L capacity) (Bay Plastics Ltd., UK). It had two sliding screens, ventilation holes (Ø 2 mm) and six entries (3 on either side) to fit feeding tubes (modified 2.0 mL centrifuge tubes, Ø 10.8 mm). b) commercially available Really Useful Boxes® of polypropylene plastic (external dimensions: 19.5 x 13.5 x 11.0 cm; 1.6 L capacity). Each box displays two handles on each side enabling a lockable top lid suitable for stacking. For suitable ventilation (Ø 2 mm) and to support feeding tubes (Ø 10.9 mm), holes were manually drilled post-purchase.

Figure 2.3 Conditions of feeding assays inside incubators. Treatment boxes were kept inside ventilated incubators (4 shelves) at 34ºC in the dark (top). Boxes were randomly scattered across shelves. Temperature and relative humidity were recorded. At the rear of each shelf, one water tub (tap water) was provided and refilled every 2 days (bottom).

2.4.2 Study 1. Results

Feeding responses under mineral diet restriction: no-choice assays can be used to reveal how feeding on a restricted diets affects overall consumption and survival. Results from no-choice assays are shown in Figure 2.4 (salts) and 2.5 (metals) and indicate the mean volume consumed per bee for each treatment and the daily survival (%) under feeding restriction. Although, no formal statistics were computed for consumption data due to low sampel size (N= 2–3 boxes/treatment), the data suggested that consumption across treatments did not differ. Yet, compared to sucrose alone (control), bees ingested slighty more of K (low and high), Ca (high), Fe (low and medium), Cu (high) and Mn (medium). Under high Mg, Zn and Cu treatments, bees apparently consumed less solution compared to sucrose alone (40–60 μL/bee over 7 days).

Survival of young workers fed single mineral diets: survival data statistics are presented in Table 2.1. The Kaplan-Meier method was used to test honey bee survival under different mineral treatments over 7 days. At the end of the experiment (day 7), more than 50% of bees were still alive for most group treatments. High Ca (5000 ppm) and Cu (500 ppm) were the exceptions yielding 63.9 % death (at day 6), and 59.0 % (at day 3), respectively. Log-rank statistics produced statistically significant differences for all, but K, Fe and Mn treatments. As expected, the hazard of dying increased under high salt/metal solutions by day 7, except for Na. Here, 16.7 % of bees confined to medium Na (50 ppm) diets were dead by day 7. Nevertheless, under high Cu (500 ppm) solutions, the risk of dying is increased such that by day 5, all the bees in this treatment were dead.

Figure 2.4. Pilot no choice feeding assays to test the experimental design and the effects of salt diet treatments on food consumption and survival in honey bees.

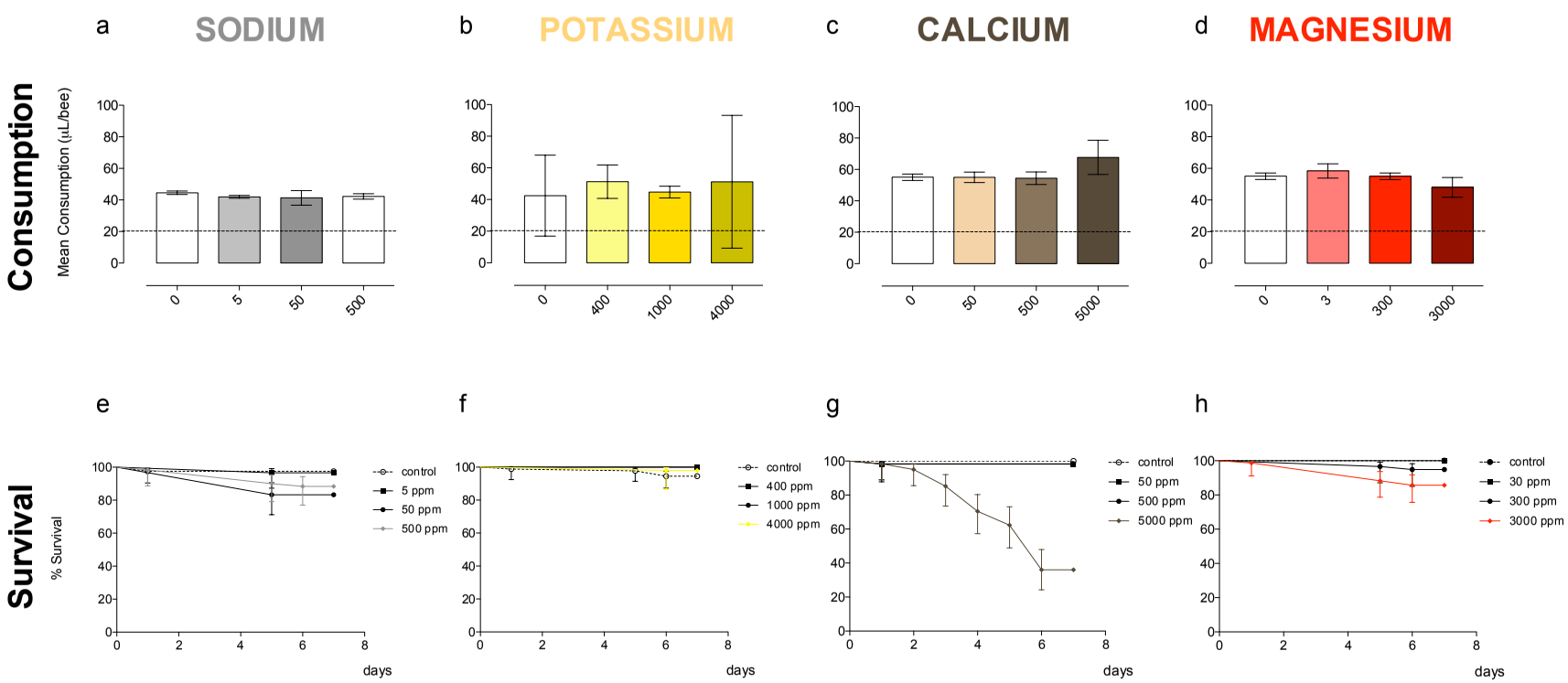

Figure 2.4. Pilot feeding assays (no-choice) to assess honey bee consumption under salt diet restriction. Bar plots **a, b, c** and **d** represent consumption (Mean±SEM) of salt treatments. Diet treatments consisted of four increasing concentrations of each salt in 1.0 M sucrose solution from control (0 ppm) to high salt. Newly emerged bees were confined to a single diet varying salt identity and concentration. All salts were provided as chloride conjugates. Each treatment included N= 2—3 feeding boxes with N= 92—50 bees/treatment. Feeding boxes were considered the experimental units for replication. Consumption measurements were conducted daily and over 7 days. No formal statistics were performed for consumption data due to low sample size. For comparison, a dashed line crossing each graph at 20 µL corresponds to 1/3 of worker crop volume reported previously. Graphs **e, f, g** and **h** indicate survival curves for each treatment (Mean±95% CI). Survival data were statistically tested using the Kaplan-meier method.

Figure 2.5. Pilot no choice feeding assays to test the experimental design and the effects of metal diet treatments on food consumption and survival in honey bees.

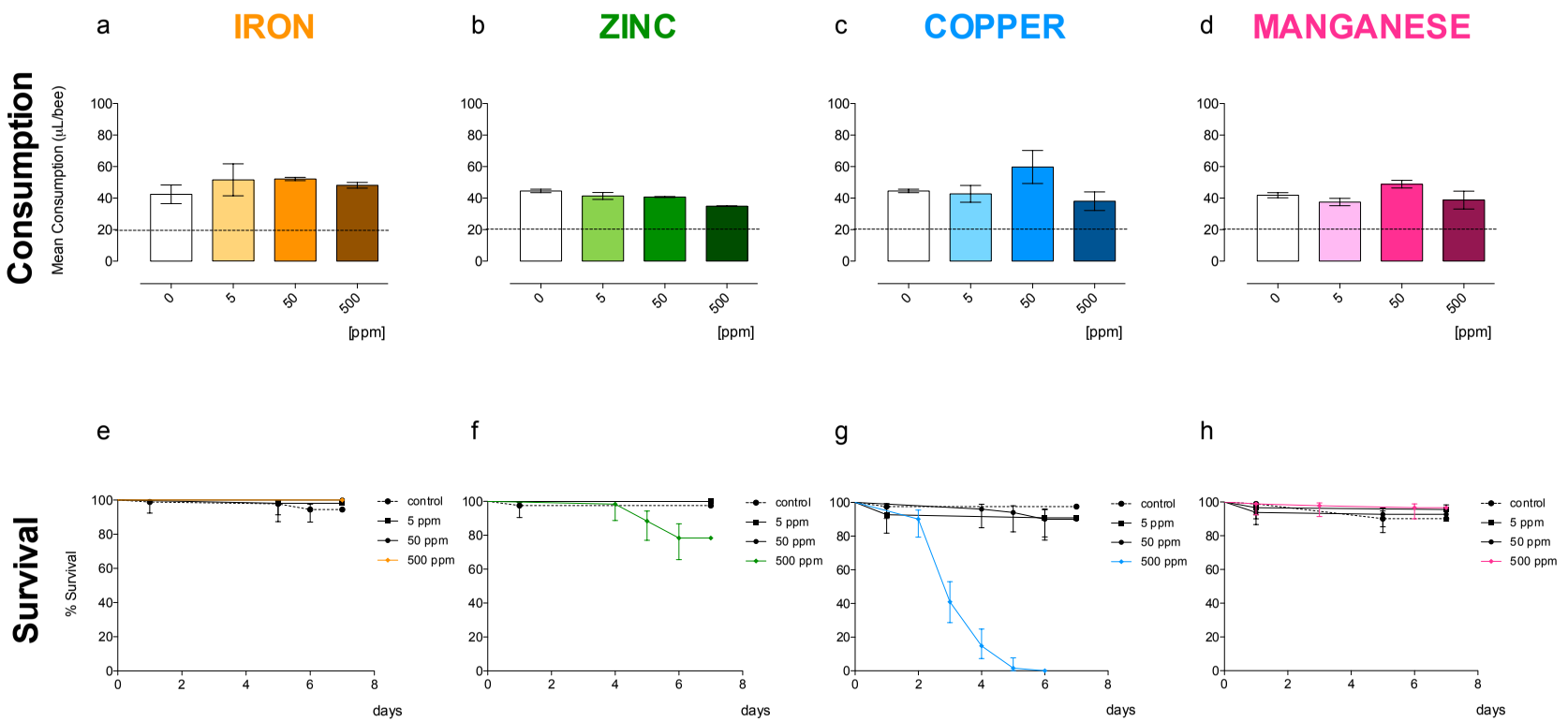

Figure 2.5. Pilot feeding assays (no-choice) to assess honey bee consumption under metal diet restriction. Bar plots **a, b, c** and **d** represent consumption (Mean±SEM) of metal treatments. Diet treatments consisted of four increasing concentrations of each metal in 1.0 M sucrose solution from control (0 ppm) to high metal. Newly emerged bees were confined to a single diet varying metal identity and concentration. Zinc and manganese salts were provided as chloride conjugates, whilst iron and copper were citrate and sulfate salts, respectively. were provided as chloride conjugates. Each treatment included N= 2—3 feeding boxes with N= 96—49 bees/treatment. Feeding boxes were considered the experimental units for replication.Consumption measurements were conducted daily and over 7 days. No formal statistics were performed for consumption data due to low sample size. For comparison, a dashed line crossing each graph at 20 µL corresponds to 1/3 of worker crop volume reported previously. Graphs **e, f, g** and **h** indicate survival curves for each treatment (Mean±95% CI).. Survival data were statistically tested using the Kaplan-meier method.

Table 2.1 Kaplan-Meier (Log-rank estimator) testing differences between survival curves of honey bees fed mineral diets over 7 days.

Source (Treatment)	Wald $\chi 2$	df	P value
Salts			
Sodium	11.3	3	**0.01**
Potassium	6.20	3	0.10
Calcium	159.	3	**<0.01**
Magnesium	21.6	3	**<0.01**
Metals			
Iron	6.00	3	0.11
Zinc	36.7	3	**<0.01**
Copper	224.	3	**<0.01**
Manganese	3.80	3	0.28

Values in bold highlight a probability value (P value) < 0.05, indicating a mean significant difference at the level of 5%.

Acrylic box design: the analysis of box designs was based on three main qualitative and informal parameters 1) manipulation and material (preparation, stacking, hygiene; 2) ventilation and tube delivery; and 3) reproducibility of measurements. Acrylic (Polymer of Polymethyl Methacrylate, PPMA) boxes were easy to wash, wipe and dry mostly due to removable front and back sliding screens (see Figure 2.2a). With external dimensions of 13 x 11 x 4 cm, these boxes were easily clustered inside the incubators and allowed fitting of up to 100 boxes inside the incubator if needed. However, these boxes were not suitable for stacking due to narrow breadth (4 cm). Also, each acrylic box was composed of four pieces: top, bottom and sides, front and back removable screens. Top screen was glued to the edges of side lateral screens. Handling older boxes of this design was more difficult, and increased the risk of collapsing and, therefore, prone to compromise replicates. The feeding tube openings and ventilation holes were manufactured and standardised across boxes, air circulation was ensured (no major condensation inside the box nor "unusual bees"[1] were found (see S2 Figure, Appendix A) and feeding tubes were inserted smoothly and did not spill often. Sliding screens had to be pushed up about 1 cm to remove dead bees by means of forceps. Care was

[1] "Unusual bees": bees with unusual looks, darker and bright possibly due to high moisture or hair loss.

imperative to prevent bees from flying out. Inserting and removing feeding tubes for measurements and diet replacement was appropriate and did not generate much nuisance nor dripping, rendering measurements systematic.

"Sandwich box design": the second box model made of polypropylene plastic was also easy to manipulate and washable, though less effective to air dry as this box does not disassemble in pieces besides the removable lid (see Figure 2.2 b). Moreover, by exhibiting handles on either side to lock a top lid, this box was less prone to dislodge and safer to handle. Its raised edges on the lid allowed effective and secure stacking, but reduced air circulation inside the boxes if ventilation holes were drilled on the top lid as well. These boxes had larger dimensions (19.5 x 13.5 x 11.0 cm) compared to the acrylic boxes, and fewer of them fitted inside the incubators. To remove dead bees, forceps were used by inserting through the feeding holes. This task was very hard and time-consuming. Feeding holes were manually adapted to the front and back sides of these commercially available boxes. This produced uneven holes (e.g. not aligned, slightly larger/smaller), rough edges around the opening and plastic debris. Inserting and removing feeding tubes was more difficult (e.g. friction), leading to frequent spilling events, most of them scarcely accountable for.

While conducting these feeding experiments, other issues were flagged and considered likely to affect response variables (e.g. consumption measurements). Factors such as the position of feeding boxes inside the incubator, the method to adjust consumption measuments for solution evaporation loss, and the reliability of consumption measurements in treatments (which induced high mortality and fewer bees per box) were, therefore, considered of particular importance to produce consistent and accurate results. These topics will be further tested in subsequente sections.

2.4.3 Study 1. Discussion

No-choice assays were used to reveal how feeding on a restricted mineral diet (above or below the optimum) affects overall consumption and survival of adult honey bees. In contrast to choice assays, confining bees to diets varying in mineral concentrations was expected to yield more conspicuous responses on bee survival under specific diets if an effect occurred. Therefore, no-choice feeding assays are considered more indicative of sublethal or lethal mineral concentrations by ingestion over a defined period.

By analysing adult bee survival and brood rearing based on bee-collected pollen, Herbert and Shimanuki (Herbert and Shimanuki, 1978) suggested that adult bees required the following mineral concentrations: K: 1,000 $mgKg^{-1}$; Ca: 500 $mgKg^{-1}$; Mg: 300 $mgKg^{-1}$; Na, Fe, Zn Mn, Cu: < 50 $mgKg^{-1}$. Therefore, I followed these recommendations and defined the medium (Md) levels similar to those proposed by Herbert and Shimanuki. My hypothesis was that, on average, bees would consume more of this diet in comparison to any other treatment as suggested by Bertrand's rule model whereby he applied the concept of dose-respose curve to micronutrients (Bertrand, 1912; Mertz, 1981) (see Figure 1.3, Chapter 1). This model suggests that at low concentrations of a tarce element, consumption is increased until an optimum, followed by a reduction as excesses may induce toxicity. Besides, and most importantly, I expected to validate whether the highest concentration (Hi) tested induced the highest mortality. If that was the case, then I could define a sublethal range of concentrations to scale up feeding assays and fine tune the study (Figure 2.4 and 2.5).

These preliminary results showed that the no choice feeding assays were effective on demonstrating the impacts of feeding mineral solutions on adult bee survival. These data also indicate that the mineral treatments within these ranges were sublethal as more than 80 % of bees were still alive at the end of the experiment (day 7), with the exception of Cu and Ca diets. The range of concentrations was within values that could be used in detailed experiments on the regulation of

feeding. For example, testing a wider range of concentrations towards the lower end of the range of concentrations for Ca, Mg, Zn, Cu and Mn, and testing higher concentrations especially for Na, K and Fe. On the premise that the optimal concentration would translate in higher consumption, I did not find any noticeable magnitude difference for increased consumption of medium concentration nor to any other diet treatment, including the sucrose control.

These feeding assays were also useful to test differences between two box designs: customised acrylic and standard plastics types. By considering parameters such as manipulation, material type, hygiene, tube delivery and reliability of recorded measurements, acrylic customised boxes were preferred (Figure 2.2 a). In contrast to plastic boxes (Figure 2.2 b - Really Useful Boxes®), acrylic boxes (Polymer of Polymethyl Methacrylate, PPMA) were, for example, more effective to wash between treatments, facilitated the removal of dead bees, delivered standardised openings to deliver feeding tubes; prevented dripping; and their compact design saved space inside the incubator due to smaller dimensions. These boxes are made of a type of Perspex that is UV resistant material (in case sterilisation is required). Only two drawbacks can be advanced regarding acrylic boxes acquisition: price and manufacture period, i.e. they cost £12/box compared to £5/plastic box), and they are custom-made, which can lead to manufacture waiting periods.

Acrylic boxes have already been used to maintain adult honey bees to study amino acid and sucrose intake regulation (Paoli *et al.*, 2014) and detection and consumption of pesticides (Kessler *et al.*, 2016). Previous studies have reported that good box designs improve cage conditions of adult bees (e.g. low mortality, low pathogen load, washable and resistant material or disposable plastic boxes) (Evans *et al.*, 2009; Huang *et al.*, 2014). Together, they recommended that disposable plastic cups/containers or a stainless steel frame with front and back acrylic screens, all exhibiting a top feeder (e.g. graduated plastic syringe) would support health and good care of adult bees in laboratory conditions. Here, I did not test these particular kinds of feeders, but their larger design means that , using them takes more time to

fill feeding tubes with diet and to measure them as more tubes must be used to provide enough diet per box.

Performing feeding assays using liquid diet treatments also revealed that evaporation losses should be tightly controlled and satisfactory surveillance. Here, (and against previous recommendations of N= 20–30 bees per box (Williams *et al.*, 2013), I used an uneven number of bees per box, ranging from 49 to 96 bees. This was not intentional at first, but ended up providing useful insights, as I observed that bee density inside the box greatly affected consumption and survival measurements. Importantly, overcrowding may prevent some bees to gain access to food. Too many bees per rearing cage also increases the humidity and condensation inside the box, induce abrasion of the bees (they lose their hairs), and compromises health (e.g. increased pathogen load), leading to more rapid mortality. Thus, using suitable number of bees per cage in laboratory settings can be critical to both the amount of food consumed and to prevent mortality duet to strees instead of the testing variable (e.g. diet).

The medium Na level (50 ppm) induced the highest mortality compared to other Na treatments (Figure 2.4 e and Table 2.1). This was not expected and, in fact, it was not clear whether this was due to diet treatment itself or other factors such as stress or starvation induced by overcrowding in feeding boxes. High mineral diet treatments were likely to increase death over time (e.g. high calcium (5,000 ppm) (Figure 2.4 g). Though the number of bees able to feed per box was decreasing, the average volume consumed by these bees increased compared to, for example, Ca and sucrose control treatments (Figure 2.4 c). This suggests two things: either the bees consumed more of high Ca diet before dying or the measurements of food consumption were no longer reliable after a time point when few bees were present in the box. This pilot indicates that when very low number of bees remain alive in a box, measuring consumption of liquid diets is no longer possible nor accurate using this experimental design and procedures. This topic is addressed later in this Chapter (refer to Figure 2.11).

Additionally, an important issue raised by these pilot data relates to controlling and adjust for evaporation losses from liquid diets. As many had previously done, consumption was measured daily as the mass difference in feeding boxes minus mass difference in mock evaporation boxes per diet treatment. In my pilot experiments, consumption values consisted of pooled averages over N= 2–3 boxes, whereas evaporation loss values respected a single measurement (N= 1 box). Even if we consider that evaporation rates for each solution are the same every 24 h, experimentally that does not occur. For example, many variables affect the variability and reliability of these measurements, including operator expertise, random spillage, feeder leakage, temperature inside the incubator, or other stochastic events. In fact, a major problem occurs downstream. It is often the case that when subtracting daily evaporation losses from consumption volumes, the final figure becomes a negative value (data not shown). Here, if negative consumption values are common because the evaporation control is greater than the measured intake of food, then the choice is to make these values '0' or analyse them as missing values. In my pilot, I replaced negative values with '0' (no consumption). In later experiments with the design of the protocol, I used these data to optimise the way that negative values in the data are handled.

Taken together, these pilot experiments indicate that the nature of treatments (liquid solutions of inorganic micronutrients), laboratory conditions (incubator design and conditions), evaporation loss adjustments, the nature of the testing variable (consumption by small insects) and the number of bees per box, are all factors likely to influence the magnitude, reliability and reproducibility of consumption measurements in feeding assays. For such reasons, I devoted time to optimising both the cage design and the data handling for the evaporation control which are reported in the following sections of the current Chapter.

2.5 Study 2. Evaluating Evaporation Rates Inside Incubator Chambers

2.5.1 Study 2. Rationale & Methods

Following the previous section, evaporation loss is one critical factor to account for in feeding assays using liquid diets. Here, I aimed to briefly test evaporation conditions inside incubators where caged bee cohorts were maintained during feeding assays. Two ventilated incubators (Sanyo MIR-553) were used and kept at 34ºC. Temperature and humidity were recorded at all times (S3 Figure, Appendix A). Each incubator exhibited four grid shelves, a fan in the middle of te rear wall and operated in dark conditions. Pipette tip racks (1 mL) were used as water tubs (Figure 2.3) to stabilise humidity within the incubator. These were placed at the centre of each shelf towards the rear wall. Tubs were filled with tap water only once at the begining of each experiment. Every 48 h over 192 h (8 days), each water tub was weighed and water mass loss recorded as the difference in weight at 48 h, 96 h, 144 h and 192 h after start. The effect of incubator, shelf position and evaporarion loss over time were assessed using two-way ANOVA statistics (GraphPad Software, Inc., Prism 5 for Mac OS X, version 5.0a, 2007).

2.5.2 Study 2. Results

The mass decrease in water tubs, used as a proxy for diet solutions, was recorded at different time points (48, 96, 144 and 192 h) to evaluate how the position of experimental boxes inside the incubator affected evaporation rates. Figure 2.6 a shows the total weight loss of water tubs displayed in every shelf (1–4) over 192 h. There was no effect of incubators on water loss as predicted. Though shelf position had a significant effect (2-way ANOVA, shelf: $F= 21.4$, $df= 3$, $P< 0.001$) and accounted for 71.6 % of total variation. Post-tests revealed that shelf 3 had the greatest effect on evaporation rates from water tubs (pairwise, Bonferroni, vs. shelf 1, $P< 0.001$; vs. shelf 2, $P< 0.001$; and vs. shelf 4, $P< 0.001$). Shelves 1 (top) and 4 (bottom) yielded a

difference in water loss between 80–90 g/48 h, shelf 2 (middle top) and shelf 3 (middle bottom) induced the lowest (50–60 g/48 h) and highest (130–145 g/48 h) losses, respectively. When testing whether evaporation rates changed over time (Figure 2.6 b), a significant effect of both time and shelf on water loss was observed (2-way ANOVA repeated measures, time elapsed: F= 55.7, df= 3, P< 0.001; shelf: F=

Figure 2.6. Testing the effect of shelf position on evaporation rates from water tubs inside incubators.

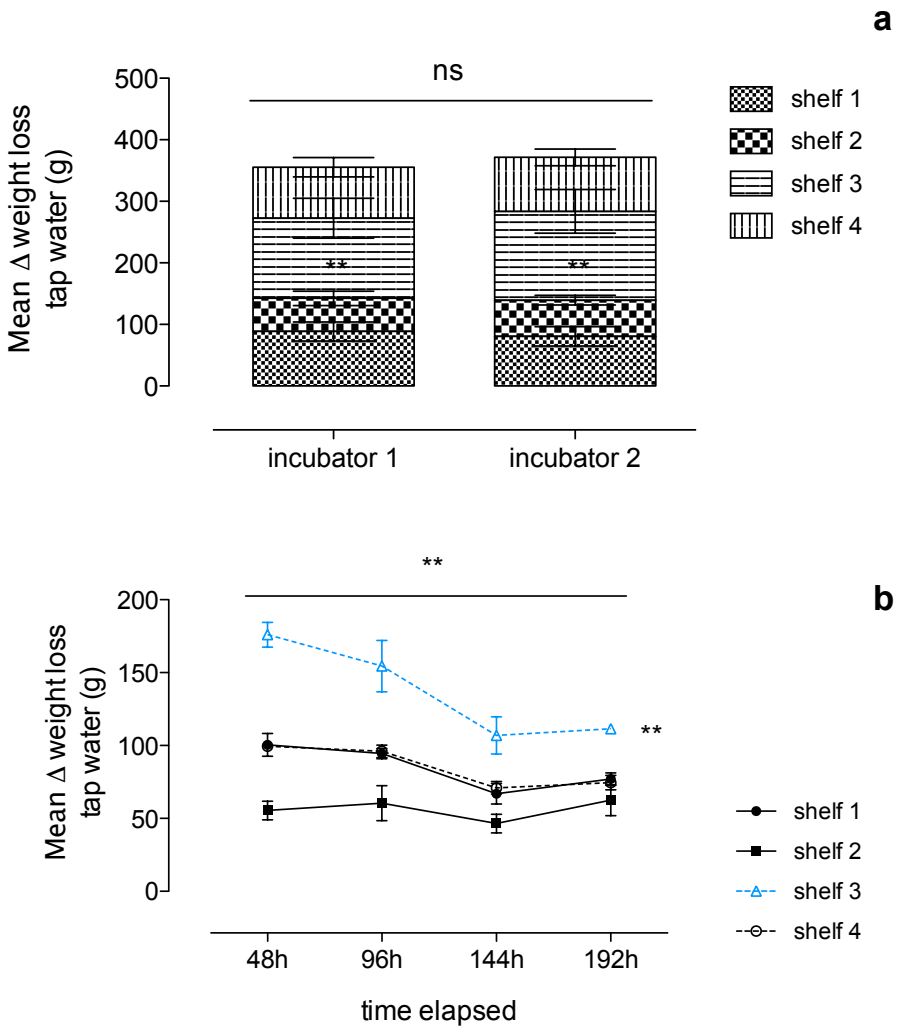

Figure 2.6. Monitoring of evaporation loss from water tubs inside incubators. Tubs (N= 1/shelf) filled with tap water were measured every two days (weight difference). Water tubes were filled with tap water once at start. Results are shown for two incubators. **Panel a)** shows total weight loss of water tubs displayed in every shelf (1—4) over 192 h. There was no effect of incubators on water loss, though shelf position had a significant effect (2-way ANOVA, shelf: F= 21.4, df= 3, P< 0.01). Post-tests showed that shelf 3 (**) had the greatest effect on evaporation rates from water tubs (pairwise, Bonferroni, vs. shelf 1, P< 0.01; vs. shelf 2, P< 0.01; and vs. shelf 4, P< 0.01). **Panel b)** displays evaporation rate over time for each shelf. Shelf and time had significant main effects on water loss (2-way ANOVA repeated measures, time elapsed : F= 55.7, df= 3, P< 0.01; shelf: F= 58.8, df= 3, P< 0.01). For both incubators, water tubs displayed on shelf 3 also had greater losses at all time points (blue-highlighted lines) compared to other positions (pairwise, Bonferroni, vs. shelf 1, P<0.01; vs. shelf 2, P<0.01; and vs. shelf 4, P<0.01). Bar and line plots show Bar Plot mean±SD.** indicate significant P value at the level of 1% produced by 2-way ANOVA statistics.

58.8, df= 3, P< 0.001). For both incubators, water tubs displayed on shelf 3 had greater losses at all time points compared to other positions within incubators (pairwise, Bonferroni, vs. shelf 1, P<0.001; vs. shelf 2, P<0.001; and vs. shelf 4, P<0.001). The highest reduction in water mass occurred after 48–144 h.

2.5.3 Study 2. Discussion

These results indicated that shelf position within the incubator may strongly affect the rate of evaporation, specifically if displayed on shelf 3 (middle bottom). The water tubs displayed on shelf 3 lost, in average, almost 2x more water compared to other shelf positions. This was expected and most likely to occur because this shelf position is directly in front of the fan that ventilates this incubator type. As such, when performing feeding experiments, care must be taken when assigning treatments to one or another shelf. By performing this straightforward study, I identified that experimental boxes with different treatments must be distributed evenly across incubator shelves and shuffled over the course of the experiment to prevent systematically increasing evaporation rates in diet treatments allocated to shelf 3, specifically. Moreover, the orientation of feeding boxes (cuboid shape) inside the incubators may be also critical to avoid overpexposure of feeding tubes facing the rear incubator side, the side most susceptible to the fan effect.

2.6 Study 3. Testing Methodologies to Correct Consumption Measurements for Evaporation Loss of Liquid Diets

2.6.1 Study 3. Rationale & Methods

When conducting feeding experiments using liquid diets, a commom methodological procedure is to add mock evaporation boxes to the experimental set up , i.e. same feeding box and feeders, but without bees, to control for solution loss by evaporation. Then, for each diet treatment, consumption measurements are corrected by subtracting mass losses recorded from these mock boxes (Williams *et al.*, 2013). This may sometimes result in negative consumption values, i.e. virtually no consumption. Here, I explored other approaches to adjust for evaporation losses when processing consumption data. I also evaluated how different approaches impact the magnitude decrease of consumption measurements. To test this, I used two-choice feeding assays using adult bees. After being assigned to experimental boxes N=30/box, bees were given a choice between sucrose (1.0 M) and sucrose enriched with salt. Distilled water was also provided. Over 6 days, two feeding tubes of fresh diet (sucrose alone, salt or water) were provided daily to each box. Feeding tubes consisted of modified microcentrifuge tubes with 3–4 holes drilled lengthwise. Treatments consisted of three increasing concentrations of salt: low + (0.22 mM), medium ++ (2.20 mM) and high +++ (22.0 mM), and included N= 10 boxes/treatment. Each treatment also included N= 4 mock evaporation boxes to control for solution loss. During the experiment, all boxes were kept inside incubators set at 34 ºC in the dark. Dead bees were removed from each box every day. Diet consumption was assessed by recording mass difference of feeding tubes every 18–26h. From mock evaporation boxes, mass difference was also recorded daily. Assuming similar variations in measurements between boxes, mock feeders were pooled together producing a single mean value per treatment solution. The mass difference was then divided by 4 to respect Δevaporation/solution/box, and hereafter referred as evaporation correction value. To test the effects of evaporation correction on solution

consumption values, I used seven different methods applied to each solution: **Method A**) no evaporation loss correction (**Δraw.consumption**), thus, raw mass consumption was used without subtracting evaporated solution; **Method B**) daily evaporation loss (g) correction (**Δraw.consumption-Δevaporation/day**). If this resulted in negative consumption values within the dataset, these were converted to zeros, assuming no consumption; **Method B1**) daily evaporation loss (g) correction (**Δraw.consumption-Δevaporation/day**), treating potential negative consumption values as missing values by experimental fail; **Method C**) mean evaporation loss (g) correction (**Δraw.consumption-Δevaporation/days**) and treating potential negative consumption values as no consumption, as in Method B; **Method C1**) mean evaporation loss (g) correction (**Δraw.consumption-Δevaporation/Xdays/solution**), assuming potential negative consumption values as missing values by experimental fail, as in Method B1; **Method D**) daily evaporation loss (%) correction [**Δraw.consumption-(Δraw.consumption*Δevaporation(%)/100)]/day/solution**), and **Method E)** mean evaporation loss (%) correction [**Δraw.consumption-(Δraw.consumption*Δevaporation(%)/100)]/*x*days,** with *x* meaning total number of days). In contrast to methods B, B1, C and C1 which correction values were used as mass losses (g), in methods D and E correction values consited of % loss converted from mass loss values (g) (Δevaporation). For example, for sucrose solution at day 1: x= initial mass (g) for sucrose solution (100% solution mass) respecting one box; y= initial mass (g) – final mass (g), thus is the Δevaporation /box; **z=y*100/x** and represents the % of evaporation loss for sucrose solution per box. The effects of correction methods on consumption data were analysed using Generalized Linear Models (GzLM). *Post-hoc* tests using Sequential Bonferroni method were applied to compare differences between correction methods (IBM SPSS Statistics for Macintosh, version 24.0, 2017).

2.6.2 *Study 3. Results*

Using a two-choice feeding assay design, I evaluated how applying different methodologies to correct for diet solution evaporation influenced the magnitude of consumption measurements. Simultaneously, I also assessed evaporation loss differenes between diet treatments varying in salt concentration. Results are shown in Figures 2.7, 2.8 and 2.9. Consumption is presented as the mass (g) difference averaged across 6 days of feeding. For each solution and treatment, values indicate the mean solution consumed (g) per experimental box (including N= 30 bees). For simplification and to assess the impact of evaporation losses in the overall magntitude consumption, results reflect mass of solution per box per treatment. Instead of reporting results for solution identity only, I also differentiated these values by the salt concentrations within each diet treatment (low +, medium ++ and high +++) to better illustrate how solutions varying in salt composition may be affected by evaporation losses. Here, seven correction methods (A, B, B1, C, C1, D and E) were employed on consumption data for each solution (sucrose alone, salt diet and distilled water) across treatments. Method A, D and E differed from the remaining; method A does not correct for evaporation losses (consumption data is used as it is, raw); methods D and E applied normalised values (evaporation loss %) to correct consumption values. Using other methods, daily (B and B1) or averaged (C and C1) mass losses were subtracted from respective consumption values. The magnitude of consumption values was barely dependent on both the evaporation adjustment method employed and diet solution (evap.method x solution, X^2: 21.3, df= 12 ,P= 0.05), but was also significantly influenced by both salt treatment and diet solution (treatment x solution, X^2: 75.1, df= 4 ,P< 0.01) (Figure 2.7 and Table 2.2). In Figure 2.7, methods A, D and E, or B, B1, C and C1 produced similar effects on evaporation adjustments of food consumption which acted independently of diet solution or salt treatment. Specifically, methods B and C (negative values transformed in zeros) induced a reduction of solution consumed by half. In contrast, methods B1 and C1 methods treated negative consumption values as 'missing data'

instead of null consumption (0 g), which were included in the analysis. This manipulation, overestimates the mean consumption of water (Figure 2.7 c). Overall, *post-hoc* tests (Figure 2.7 d) revealed that method A (raw measurements) was statistically different from all the other methods, as expected. Also, in contrast to A, D and E, methods B, B1, C and C1 produced negative consumption values after correction of solution loss by evaporation (Figure 2.8). In Figure 2.9 are shown the mass losses from solutions pertaining to mock evaporation controls, either as absolute mass solution loss (a, b), or as relative normalisation for % losse (c, d) for comparison. Absolute mass loss to evaporation was applied in methods B, B1, C and C1; normalised relative values were used in methods D and E to correct values. Here, measurements from four mock boxes were pooled together and a single value is presented per solution per box. No formal statistical analysis was conducted due to low sample size. These results demonstrate that changes in mass solution due to evaporation did not differ greatly between sucrose only diets or those supplemented with salt. In contrast (and as expected), distilled water experienced higher mass losses. Mean daily losses for sucrose, salt diets and water were ~ 6 %, ~ 6 % and 10 %, respectively. Figure 2.9 (b, d) illustrate daily losses over time for salt diets only. As expected, mass loss to evaporation followed a similar pattern and varied between 0.2–0.6 g or 3–8 % every day.

Table 2.2. Generalized Linear Models testing the effects of evaporation correction methods (A, B, B1, C, C1, D and E), salt treatment (+, ++, +++) and diet solution (sucrose, salt, water) on consumption measurements in two-choice assays.

Source	Wald $\chi 2$	df	P value
(Intercept)	10,993.	1	**<0.01**
evaporation.method (evap.meth)	943.	6	**<0.01**
treatment	0.39	2	0.82
diet solution (solution)	396.	2	**<0.01**
evap.meth x treatment	3.60	12	0.99
evap.meth x solution	21.3	12	**0.05**
treatment x solution	75.1	4	**<0.01**
evap.meth x treatment x solution	3.80	24	1.00

Values in bold highlight a probability value (P value) < 0.05, indicating a mean significant difference at the level of 5%.

Figure 2.7. Effect of evaporation loss correction methods on the decrease magnitude of consumption measurements of salt diets in choice feeding assays.

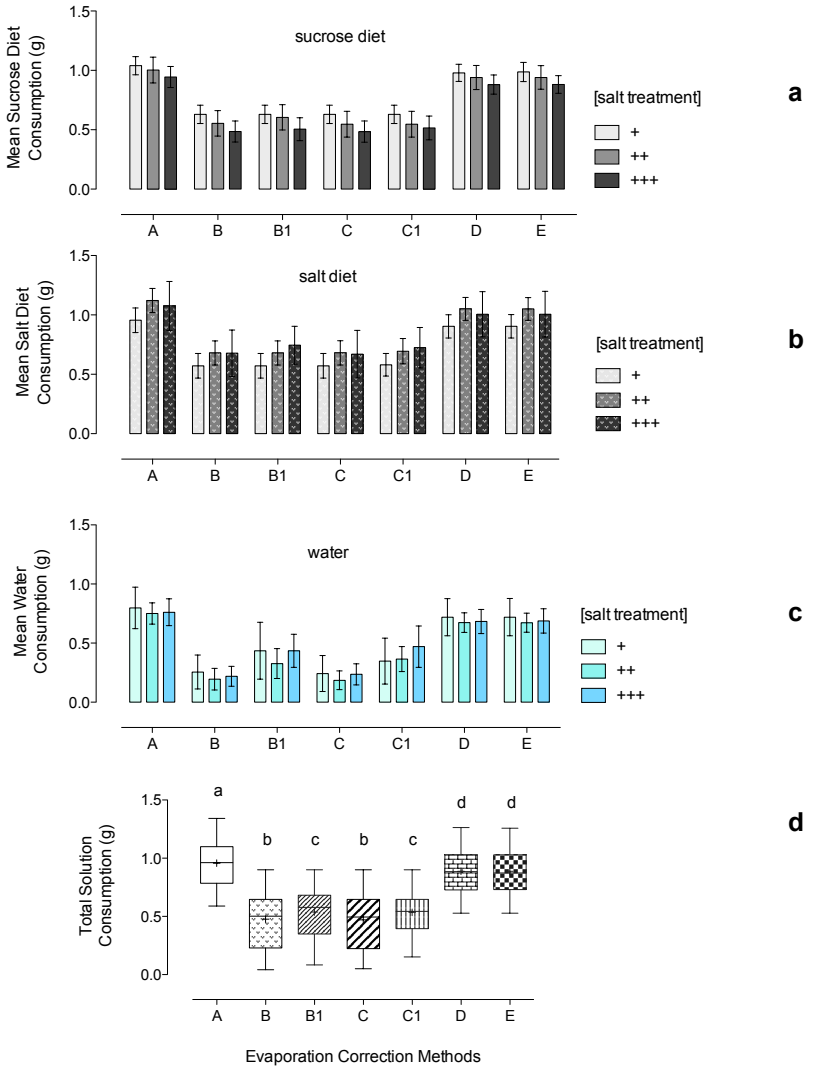

Figure 2.7. Evaporation correction methods applied to consumption measurements of liquid diets used in two-choice feeding assays. Three solutions were delivered per feeding box: sucrose, salt and water. Feeding was recorded for 6 days, and treatments varied in salt concentration from low +, medium ++ and high +++ (N= 10 boxes/treatment). **Panels a, b** and **c** show solution consumption (mass, g) after deducing evaporation loss (correction values) according to one of seven methods (A, B, B1, C, C1, D and E). A: no evaporation loss correction (Δraw.consumption); B and B1: daily evaporation loss (g) correction (Δraw.consumption-Δevaporation/day) with zero consumption either accepted or not*; C and C1: mean evaporation loss (g) correction (Δraw.consumption-Δevaporation/6days) with zero consumption either accepted or not*; D: daily evaporation loss (%) correction (Δraw.consumption-Δevaporation/day); and E: mean evaporation loss (%) correction (Δraw.consumption-Δevaporation/6days). Consumption (Mean±95% CI) indicate mean was obtained by weigh difference of each solution every 18—26h over 6 days. **Panel d** indicates the overall magnitude decrease in consumption measurements after using correction methods. Box-plots indicate the minimum and maximum range of values for each independent treatment; (—) and (+) indicate median and mean, respectively. GzLM analysed the effects correction method, treatment and solution on consumption magnitude decrease. Different letters in box plots suggest statistical differences ($P< 0.05$). *If negative values emerged after evaporation loss corrections, consumption values were assumed either as zeros (no consumption) or as missing values (experimental fail).

Figure 2.8 Percentage of negative consumption values produced after correcting for solution evaporation loss using different methods (A, B, B1, C, C1, D and E).

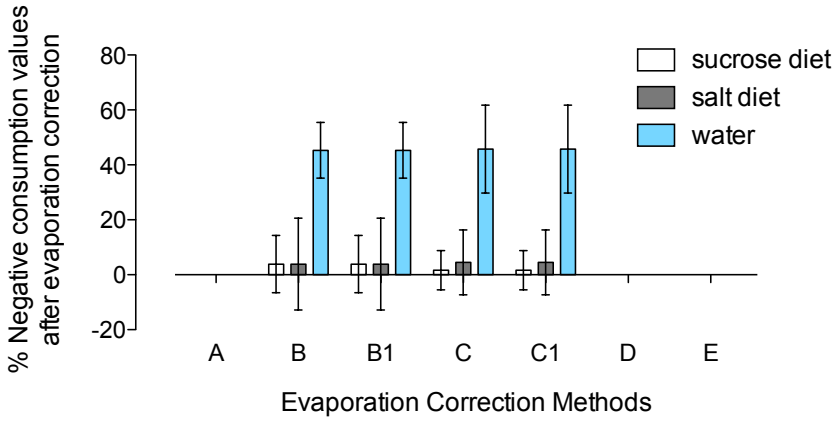

Figure 2.8 Bar plot (Mean±95% CI) illustrates the absolute percentage of negative values produced after applying correction calculations to consumption measurements for each solution delivered in feeding boxes (two-choice assays). Correction calculation methods: A) no evaporation loss correction (Δraw.consumption); B and B1) daily evaporation loss (g) correction (Δraw.consumption-Δevaporation/day) with zero consumption either accepted or not*; C and C1) mean evaporation loss (g) correction (Δraw.consumption-Δevaporation/6days) with zero consumption either accepted or not*; D) daily evaporation loss (%) correction (Δraw.consumption-Δevaporation/day); and E) mean evaporation loss (%) correction (Δraw.consumption-Δevaporation/6days). *If negative values emerged after evaporation loss corrections, consumption values were assumed either as zeros (no consumption) or as missing values (experimental fail).

Figure 2.9 Solution loss from liquid diets in mock boxes used to control for diet evaporation in feeding assays

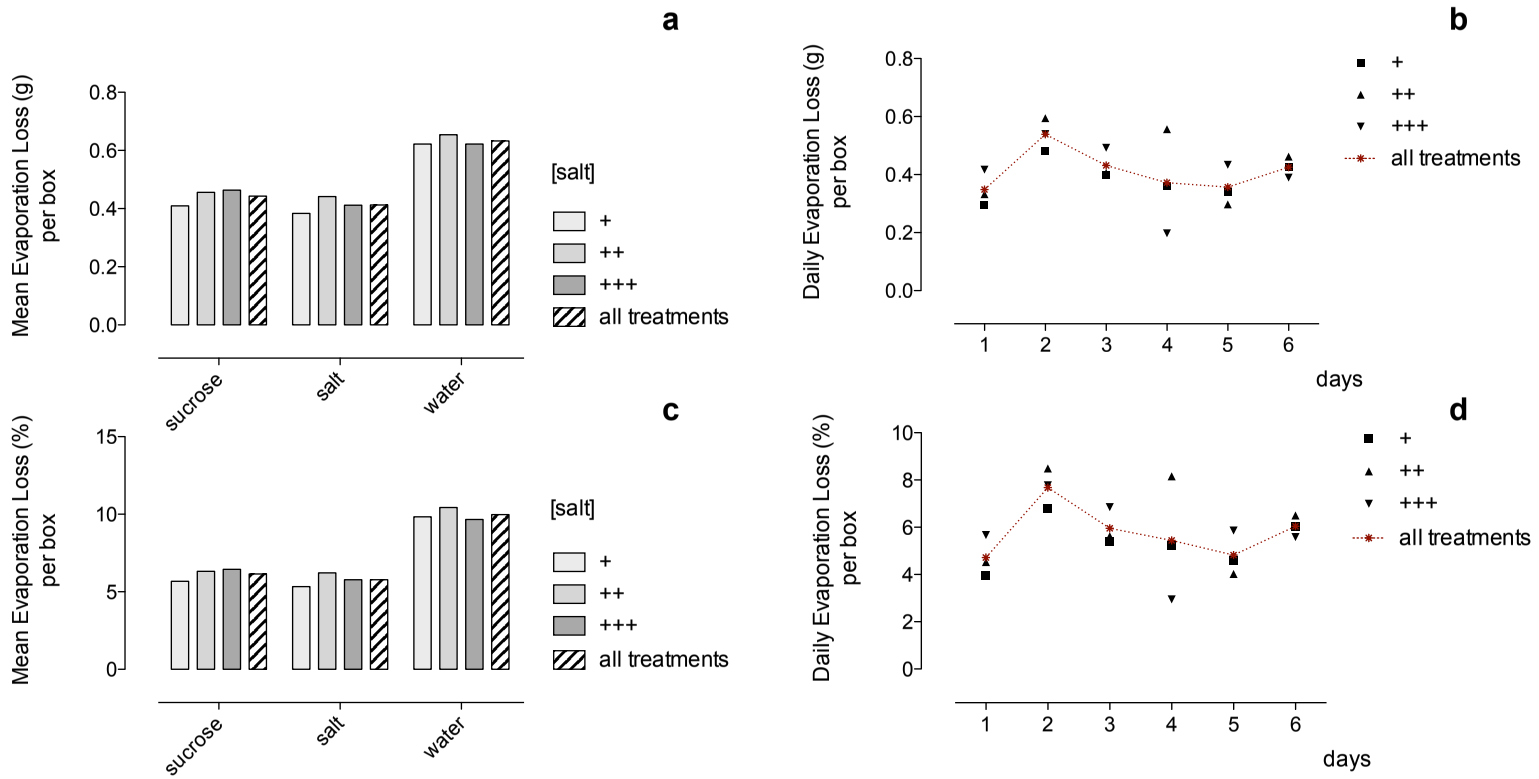

Figure 2.9 Solution loss from control evaporation boxes used in two-choice feeding assays conducted over 6 days. Solutions: sucrose (1.0 M) solution, sucrose enriched with salt and distilled water. Treatments varied in salt concentration: low +, medium ++ and high +++. Each treatment included N= 4 mock boxes with two feeding tubes per solution. For each solution box measurements were pooled together and, therefore, a single mean evaporation loss per box over 6 days is depicted. **Panels a** and **c** show the mass loss over 6 days for each solution across salt treatments. Bar plots show the magnitude of solution loss in two different units: mass (g) and % loss. The latter was normalised from initial solution mass and mass differences over 6 days. **Panels b** and **d** show the daily mass loss by evaporation for salt solutions only. Line plots show evaporation loss over time across treatments. All treatments represent the grand mean for each solution and is depicted as a dashed red line for relative comparison (b and d).

2.6.3 Study 3. Discussion

Following preliminary feeding assays (Study 1 in section 2.2), I realised that accounting properly for evaporation losses form liquid diets is critical to obtain reliable results and reproduce experiments. Besides, it has been common practice in similar experimental set ups that consumption measurements are further processed by simply subtracting mean evaporative loss of a solution without considering how it may affect measured values (Williams *et al.*, 2013; Huang *et al.*, 2014; Paoli *et al.*, 2014; Kessler *et al.*, 2016). Although further details are often omitted, these procedures are likely to result in a fraction of negative consumption values, which means fewer data or misleading data when the values are entered as null values. Here, I showed that methods B/B1 and C/C1, most traditionally used, are prone to generate negative values in the dataset (2–10% diet solutions and > 40 % distilled water (Figure 2.8). In fact, methods B1 and C1 were tested to explore other insights to solve negative consumption values that are likely to occur. As shown, these procedures can virtually affect the magnitude of mean consumptions, especially in cases exhibiting >20% negative consumption values after correction (e.g. water). Thus, dealing consistently with potential negative values is critical to analysis and interpretation of the results. Moreover, considering that the magnitude of consumption by a single honey bee is low (< 1 mL), working on slightly higher ranges of magnitude is preferred. Therefore, either methods D or E provide a better correction methodology for experiments of this kind. Compared to method A, which reflects raw consumption *per se*, these methods do not have a strong influence the magnitude of consumption values. As such, large changes in magnitude after evaporation adjustments are not anticipated. This is because experiments are conducted consistently and experimental designs and laboratory conditions across treatments are maintained constant as much as possible. Furthermore, these methodologies do not manipulate data, but rather use consumption and evaporation loss measurements to generate a relative normalised value to adjust the whole dataset.

In regards to the daily vs. mean evaporation losses, current data suggest that daily losses may be subjected to higher experimental error and increase variability. Mock boxes are external controls and, as such, are more likely to be affected by external factors (e.g. position inside the incubator). To mention evaporation losses between different diets, I predicted that salt diets would show lower rates of evaporation loss compared to sucrose solution alone. Salt in water is known to reduce evaporation rates of solutions (Al-Shammiri, 2002). This was not the case here as all diets, except water, showed similar evaporation rates, possibly because salt concentrations were still very low (ppm). In summary, feeding experiments using liquid solutions require evaporation controls to account for solution losses. The single most important criterion is to use a correction method that does not require data manipulation nor produce negative consumption values. Here, methods D and E, which applied normalised percentage evaporation loss acquired from absolute mass losses for each solution were proven to be most effective and attained the best results.

2.7 Study 4. Testing the Impact of Diet Delivery Regime on Diet Consumption

2.7.1 Study 4. Rationale & Methods

Choice feeding assays are often employed to assess animals' dietary self-selection and preferences. These experimental designs involve at least two food choices. As illustrated in previous sections (Study 3) and following the designs of feeding cages (section 2.2, Figure 2.2), feeders are usually delivered on each side of the rearing cage. Many studies in honey bees indicate that bees can use spatial orientation for homing and food location in the field. Bees can establish relationaships between environmental metrics such as direction and distances using the sun compass and landmarks, and then retrieve memories from learnt flights, even in the absence of sun (von Frisch, 1967; Wehner, 1992; Wehner, Michel and Antonsen, 1996; Menzel *et al.*, 2005). Others prostulated that bees can sense the

magnetic fields and orientate via magnetoreceptors (iron granlues) present in the abdomen (Kuterbach *et al.*, 1986; Liang *et al.*, 2016), though this is still not confirmed.

Because diet solution tubes in the current experimental design (section 2.2, Figure 2.2) can take different locations within the feeding boxes, I aimed to briefly investigate the effect of a potential spatial bias on food preference and total consumption within the standard rearing cages. Using two-choice feeding assays, adult honey bees (N= 30/box) were randomly assigned to feeding boxes and offered a choice between alone sucrose (1.0 M) or sucrose laced with low salt (44 mM). Distilled water was also provided and recorded, but was not assessed for position. One tube of water was delivered in each side of the box in a fixed positon (Figure 2.10 a). Feeders consisted of adapted microcentrifuge tubes with 3–4 holes drilled lengthwise to allow lapping (N= 2 feeding tubes/diet). These were replaced by fresh ones every day. Dead bees were recorded daily and removed from the box. Mock evaporation boxes (N= 2/treatment) were also included. All experimental boxes were kept inside an incubator at 34 ºC in the dark and shuffled every day. Solution consumption was obtained by measuring the daily change in weight of feeding tubes over 16 days.

To ascertain an effect of tube location on feeding (preferences and total consumption), I tested two tube set-ups: side-by-side and crosswise (N= 7 boxes/treatment) (Figure 2.10 a). In side-by-side regime, diet tube pairs switched sides across the box every day, i.e. on day 1, one pair of diet tubes (1A and 2A) was displayed on the left-side of the box, whilst the other pair (1B and 2B) was displayed on the right, and *vice-versa* on day 2. This method also intended to measure the effect of moving the tubes around within feeding boxes. In the crosswise treatment, diet tubes were counterbalanced across the box in a fixed, but alternated position in relation to its match. For example, one side of the box exhibited a single tube per diet (tubes 1A and 1B) and matched their pair (tubes 2A and 2B) diagonally on the opposite side. The effects of tube position on consumption responses and diet preferences were analysed using Generalized Linear Models (GzLM) (IBM SPSS Statistics for Macintosh, version 24.0, 2017).

2.7.2 Study 4. Results

I tested whether the position of diet tubes within feeding boxes affected preference and total consumption between two sucrose diet solutions (salt-free and salt-enriched). Figure 2.10 shows data for the total consumption of each diet solution (Figure 2.10 b) and total consumption per treatment (except water) (Figure 2.10 c) under side-by-side or crosswise tube set-ups. Consumption responses were significantly affected by diet solution (X^2: 135., df= 1, P< 0.001) and tube position (X^2: 326., df= 2, P< 0.001). As expected, bees demonstrated a preference for low salt diet over sucrose alone and this pattern was similar in both tube treatments (side-by-side and crosswise) (Figure 2.10 b). Each bee consumed on average 0.01g more of low salt diet than sucrose solution alone. Tube position had a strong effect on the magnitude of total solution consumed (χ^2: 24.8, df=1, P<0.001). The fixed counterbalanced tube position reduced the total food consumption by 0.02 g/bee compared to side-by-side tube position (Figure 2.10 c). Additionally, total consumption was significantly dependent on day, box and tube position (days x box x tube position: χ^2= 2,978. x 10^{12}, df= 30, P< 0.001) (S4 Figure, Appendix A). Every day (over 16 days), honey bees consistently consumed more solution in the side-by-side treatment, compared to the crosswise tube set up (S4 Figure, Appendix A).

Figure 2.10 Effect of feeding tubes' position on the magnitude of diet consumption in choice feeding assays using honey bees.

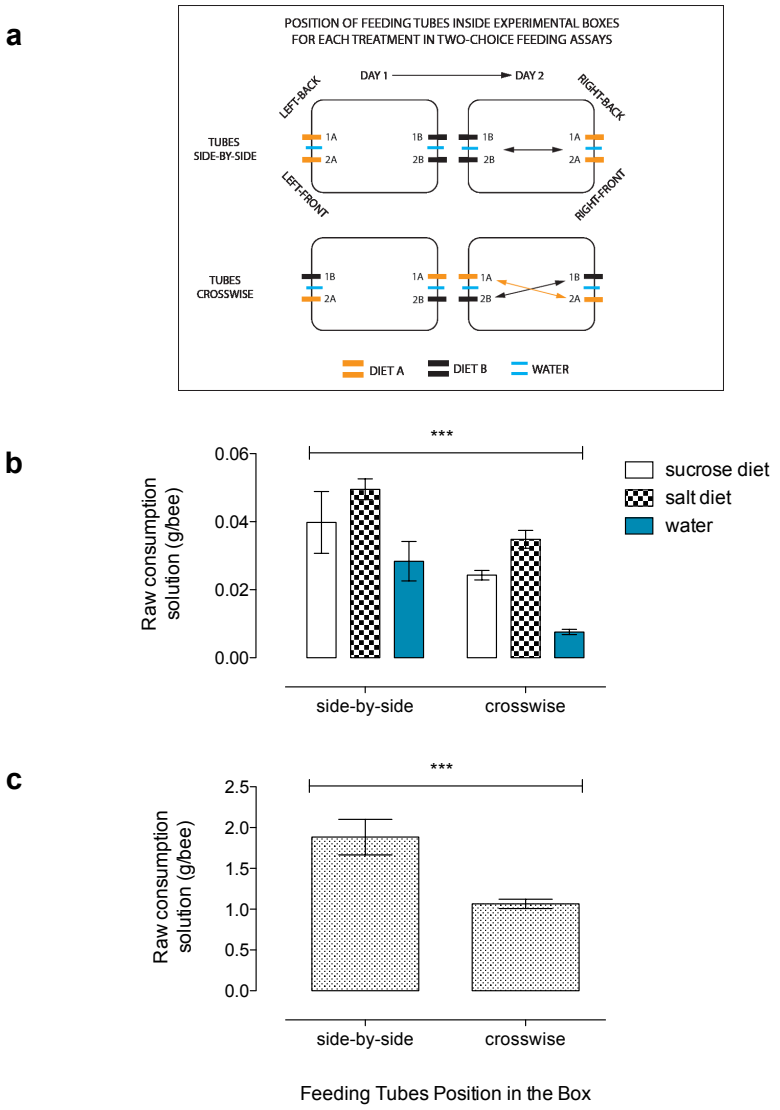

Figure 2.10 Testing feeding tubes' position on the magnitude and lateral bias of diet consumption. Adult bees were offered a choice between sucrose (1.0 M) and sucrose laced with salt over 16 days. Distilled water was also provided (2 tubes/box); water tube position did not change; water tubes displayed on the top of diet tubes. Feeders consisted of modified microcentrifuge tubes with 3-4 holes drilled lengthwise (∅ 2mm) to allow lapping. Each diet was delivered in two tubes (~5mL/diet) every day. Panel **a)** illustrates treatment design varying in feeders position (top view) within experimental boxes: side-by-side or crosswise. In side-by-side regime, diet tube pairs (e.g. sucrose or salt) switched sides across the box every day, whereas in crosswise treatment, diet tubes were counterbalanced in a fixed position. Diet solutions were replaced by fresh ones every day. Each treatment included N= 7 boxes with N= 30 bees/box. Consumption was recorded by solution change in weight and divided by the number of bees per box each. Bar plots (Mean ± 95%CI) depict **b)** the mean (raw) consumption (g/bee) for each solution, and **c)** and total (raw) consumption (g/bee) over 16 days for both treatments (tube position). Consumption data here was not corrected for evaporation (Evaporation Correction Method A - raw consumption - in section 2.3) nor density.

2.7.3 *Study 4. Discussion*

When designing feeding experiments, several aspects of bee biology and behaviour must be taken into careful consideration. For instance, these studies can provide insights into taste physiology, food preferences, intake and nutritional regulation in honey bees. Studies using laboratory feeding assays often disregard mentioning how feeders are positioned and manipulated over the course of the experiments. Therefore, one of the aims here was to assess whether tube position within the feeding box affected the magnitude of food consumption and food preferences. The second aim was to ascertain the effect of moving the tubes around every day, i.e. by shifting diet tubes laterally every day in the side-by-side set-up. I found that tube position and location had a significant impact on the magnitude of measured consumption, but not on feeding preferences (Figure 2.10 b and c). This suggests that bees were able to self-select their preferred diet solution in both tube set ups tested.

This study was initially thought to test the influence of tube position on the potential lateral bias of feeding responses, i.e. a bias implying that bees would prefer a diet solution on the left simply because it was delivered on the left side of the box. Yet, soon I realised this experemiental design did not really addressed that question as I was both lacking other tube combinations, and shuffling boxes laterally and across shelves due to reasons discussed in Study 2 (section 2.3) to minimise evaporation bias in different shelf positions (Figure 2.6). For instance, one day the front screen of one box was facing the door of the incubator, the next day was facing the rear of the incubator.

Overall, similar feeding preferences were maintained in both tube delivery regimes (Figure 2.10 b) (see study 2, section 2.3). However, by testing two tube set-ups to deliver a choice between diets, I could see an influence on the daily solution consumption (S4 Figure, Appendix A), and consequently on the overall magnitude consumption (Figure 2.10 c). In contrast to crosswise fixed tube position, bees in the side-by-side tube treatment consumed more solution (0.02 g/bee). In both scenarios,

bees were able to regulate food intake and choose their preferred diet (low salt), though the differences between tube set up treatments may indicate that bees were better able to regulate food intake when the location of food was always in a fixed position (e.g. crosswise tube position). In addition, this consumption difference between treatments could result, in part, from experimental error. By shifting tubes daily, as in the side-by-side tube set up, measurments are more prone to error from tube spillage. Shifting the pair of diet tubes every day was first intended to randomise feeding and minimise the effect of learning tube position. Bees can learn through taste by both detecting chemicals in food and associating the postingestive consequences of consuming, for example, a new food type (Ayestaran, Giurfa and De Brito Sanchez, 2010; Wright *et al.*, 2010; Scott, 2011). As such, two opposite physiological and behavioural outputs can emerge: conditioned taste aversions or preferences (Lee and Bernays, 1990; Scott, 2011), which are regulatory mechanisms for food intake (Bernays and Simpson, 1982; Trumper and Simpson, 1994; Chapman and de Boer, 1995). Food (taste) aversions are far well-studied than food preferences in bees, especially for mineral salts (Ayestaran, Giurfa and De Brito Sanchez, 2010; Wright *et al.*, 2010; Wright, 2011). In contrast to olfactory conditioning whereby taste functions as the unlearned reinforcer as a sugar reward (Bitterman *et al.*, 1983; Simcock *et al.*, 2017). Using this experimental design, I envisaged that free-flying bees, exposed to two food choices over an extended period (days), were likely to learn to associate tube position with food value and palatability. A learning effect on feeding responses is, thus, one possible mechanism to regulate food and, possibly salt intake, but should not be corrected for in these experiements as it is a mechanism governing food intake.

In fact, the depression in total food intake observed in the crosswise tube regime, along with low water consumption and less measurement variablity (narrower error bars) (Figure 2.10 c) may indicate that bees learn better the location of a specific feeder tube in the dark. Honey bees learn to navigate between foraging routes and their colony by integrating mostly visual information gathered during flight in daylight (e.g. patterns, landmarks); for further details refer to (Wehner,

Michel and Antonsen, 1996; Hempel De Ibarra, Langridge and Vorobyev, 2015). Bees might use spatial orientaion in the dark, as they must navigate within the colony and rely on senses such as mechanoreception sensing (e.g. touch, vibrations, gravity), olfaction (e.g. pheromones) and hearing (e.g. vibrations sensed by the Johnston's organ on the antennae) (Dyer, 1996; Srinivasan and Zhang, 2003; Tsujiuchi *et al.*, 2007; Warrant, 2008).

Altogether, these results demonstrate that tube position and methodologies used in two-choice feeding assays can affect the overall magnitude of the measured amount of food eaten over a 24 h period. Both tube treatments were effective on assessing food choice preferences, as bees consumed more of the low salt choice compared to sucrose alone. Compared to the side-by-side set up, the crosswise tube regime seems to be advantageous from the perspective that it does not require tube shifting and, thus, can be less prone to experimental variability and error measuremetns. Yet, it yielded lower magnitude comsumption measurements, which can pose a problem when bees are fed treatments that induce high mortality. This is because the number of bees in each feeding box affects the measurement of food consumption (Study 1, section 2.2, Figures 2.4 and 2.5). The consumption data presented here was not corrected for evaporation loss of solutions (Method A discussed in Study 3, Figure 2.7) and, thus these figures show even a slightly higher magnitude than they would if fully processed. Feeding treatments using honey bees can be very labour-intensive. I had a very small time window during that season (3–4 months) to collect data. At the time of this preliminary study, other feeding experiments using side-by-side tube set up were being executed. Therefore, for later comparison of results I decided to keep using this methodology in the forthcoming studies (Chapter 4). If I were to start from the beginning I would not shift diet tubes across the box every day or would adopt the crosswise tube set up instead.

2.8 Study 5. Testing the Number of Bees per Feeding Box on the Reliability of Consumption Measurements

2.8.1 Study 5. Rationale & Methods

In our lab, when conducting feeding experiments, we record consumption by weighing feeding tubes daily over the course of the experiment using an analytical balance (QUINTIX 64 1S, Sartorious, Ltd.). Its lower limit readability is 0.10 mg, which can translate to ~100 μL of water. An adult honey bee may consume on average between 30-60 μL of sucrose solution (1.0 M) per day. Worker bee survival in lab conditions can be ensured by providing sufficient sucrose solutions at 30 % w/v (Barker and Lehner, 1978; Brodschneider and Crailsheim, 2010). About 16 μL of 30% w/v sucrose solution per bee per day satisfies individual bees' needs (Brodschneider and Crailsheim, 2010), while 20 μL is 1/3 of its crop capacity (Núñez, 1966). As shown above, I found that measuring the amount of food eaten by an individual bee is subject to high variability because the amount eaten is hard to measure in this particular experimental regime due to the evaporation loss that may occur. For example, in experimental boxes containing ~30 bees/box on day 1, I could have few bees surviving towards the end of the experiment depending on whether the treatment induces high mortality or not.

To show the effect of the number of bees in the experimental cages on the estimation of the amount of food consumedd per bee, I used no-choice feeding assays providing sucrose solution only. Newly emerged honey bees were collected and assigned to feeding treatments varying the number of bees per box: #0, #1, #3, #6, #9, #12, #15, #18, #21, #24, #27 and #30 bees (N= 5 boxes for #1 and #3 bees' treatments; N= 3 boxes for the remaining; and N= 2 for #0 which respected mock evaporation boxes) (see S5 Figure, Appendix A). Two feeding tubes of sucrose solution (1.0 M) were delivered to bees to measure consumption and prevent starvation. No distilled water was provided. Mock boxes to control for sucrose solution evaporation were recorded over 9 days. Consumption of sucrose solution was obtained by weight loss

of feeding tubes/box every 18–26h period over 5 days. To adjust for sucrose solution losses by evaporation, I employed two methods (methods E and C1) to address potential differences as discussed in a previous section in this Chapter (Study 3, section 2.4). In method C1, as previously described, the mean evaporation loss (g) is subtracted to raw consumption measurements (**Δconsumption-Δevaporation /5days**) and treats negative consumption values as missing data. Method E subtracts a normalised mean loss (%) ([**Δraw.consumption-(Δraw.consumption* Δevaporation(%)/100)]/5days**). Solution mass (g) was then converted to volume by multiplying density factor (1.12 gmL^{-1} sucrose solution – experimental mean value).

Statistical analysis using Generalized Linear Models were used to evaluate the effects of decreasing numbers of bees per box on the magnitude of solution consumption. The effect of evaporation correction methods was also included in the model (IBM SPSS Statistics for Macintosh, version 24.0, 2017).

2.8.2 Study 5. Results

I tested how measurements from boxes exhibiting single or very low number of bees influenced the magnitude and reliability of consumption measurements in this feeding designs. Figure 2.11 shows how the number of bees in a box affected the measurement of the amount of food consumed using two different approaches reported in a previous section within this Chapter (section 2.6, Study 5).

As predicted, the estimate of the total amount of food consumed increased in a way that was proportional to the number of bees per box (Figure 2.11 a, b, d, e) (GzLM: treatment: X^2: 25,355., df= 10, P< 0.001). The evaporation method also had an influence on this value (evap.method: X^2: 217., df= 1, P< 0.001). From the analysis of Figure 2.11, it is clear that consumption for treatment #1 (1 bee/box) is correlated to the assumed readibility of the balaced used (0.10 mg ~ 0.11 mL sucrose solution) after evaporation adjustment using method C1 (Figure 2.11 e). However, this is not true for method E (Figure 2.11 b). In fact, for the same treatment (#1 bees/box) there was a decrease in absolute value of 2.6x from method C1 to method E corrections

I next analysed the data for the mean daily consumption as shown in Figure 2.11 a and d. Besides the exceptionally high consumption for all treatmens in day 4, daily consumption was not affected by treatment. In most of the days, the mean consumption for treatments #1, #3, #6, #9 using method C1 was measured near the lower detection threshold (0.10 mg ~ 0.11 mL sucrose solution), but not in method E-corrected treatments. Again, the later method seems to contribute to slightly higher magnitude values. When analysing the data for the amount of food consumed per bee, there was a clear effect of the number of bees per box on the measured value for food consumption (evap.method x treatment: X^2: 2,023., df= 10, P< 0.001; evap.method: X^2: 184., df= 1, P< 0.001; treatment: X^2: 6,519., df= 10, P< 0.001). The effect was not observed for boxes that had more than 3 bees (Figure 2.11 c and f). Boxes with 3 bees or less produced values per bee that were greater than all the other treatments. Additionally, treatments containing 1 single bee (treatment #1) or 3 bees/box demonstrated greater variability (wider interquartile range reported) and were above the threshold of 0.1 mL (red dashed line). This suggests one of two scenarios: either single bees or very small cohorts of bees in this experimental set up ingest more sucrose solution or (and more likely) this experimental design cannot be used reliably for cohorts of < 3 bees per box. The method of adjustment for evaporation also had a strong effect on data for < 3 bees per box. I found up to three orders of magnitude increase in consumption per bee using method E in treatments #1 and # 3. In average, each bee was estimated to have consumed ~40–50 μL of sucrose solution over 5 days. Yet, bees in treatment #1 were reported to ingest on average ~300 μL (method E). This suggests that each bee in treatment #1, for example, consumed on average 7.5x more than a single bee in larger cohorts, which consumed ~50 μL (treatment#30, method E). In method C1, this pattern was similar, though the magnitude difference was lower (x2.5 consumption than bees in treatment #30).

Each box was taken as a unit replicate, and assuming no differences within the same treatment. Therefore, consumption for single treatments was averaged across boxes (N= 3–5/treatment). To assess whether there was an effect of box on

consumption measurements, I tested the factorial effects of boxes and treatments. As anticipated, significant main effects were only reported for treatment (GzLM: treatment: X^2: 1351., df= 10, P< 0.001), as second order interaction yielded non-significant (box x treatment: X^2: 23.7., df= 22, P= 0.36). *Post hoc* comparisons were computed to verify group differences for total consumption/box. As predicted consumption per box was greater for treatment #30 (pairwise SeqBonf vs. #1, P<0.001; vs. #3, P<0.001; vs. #6, P<0.001; vs. #9, P<0.001; vs. #12, P<0.001; vs. #15, P<0.001; vs. #18, P<0.001; and vs. #21, P<0.001). But, when these same comparisons were tested for total consumption/bee, each bee in treatment #30 consumed on average slightly less than single bees in smaller cohorts (pairwise SeqBonf vs. #1, P<0.001; vs. #3, P<0.001; vs. #6, P<0.001; vs. #9, P<0.001; and vs. #24, P<0.001).

Figure 2.11 **Effect of the number of bees per feeding box on the reliability of sucrose consumption measurements after evaporation adjustments by two correction methods.**

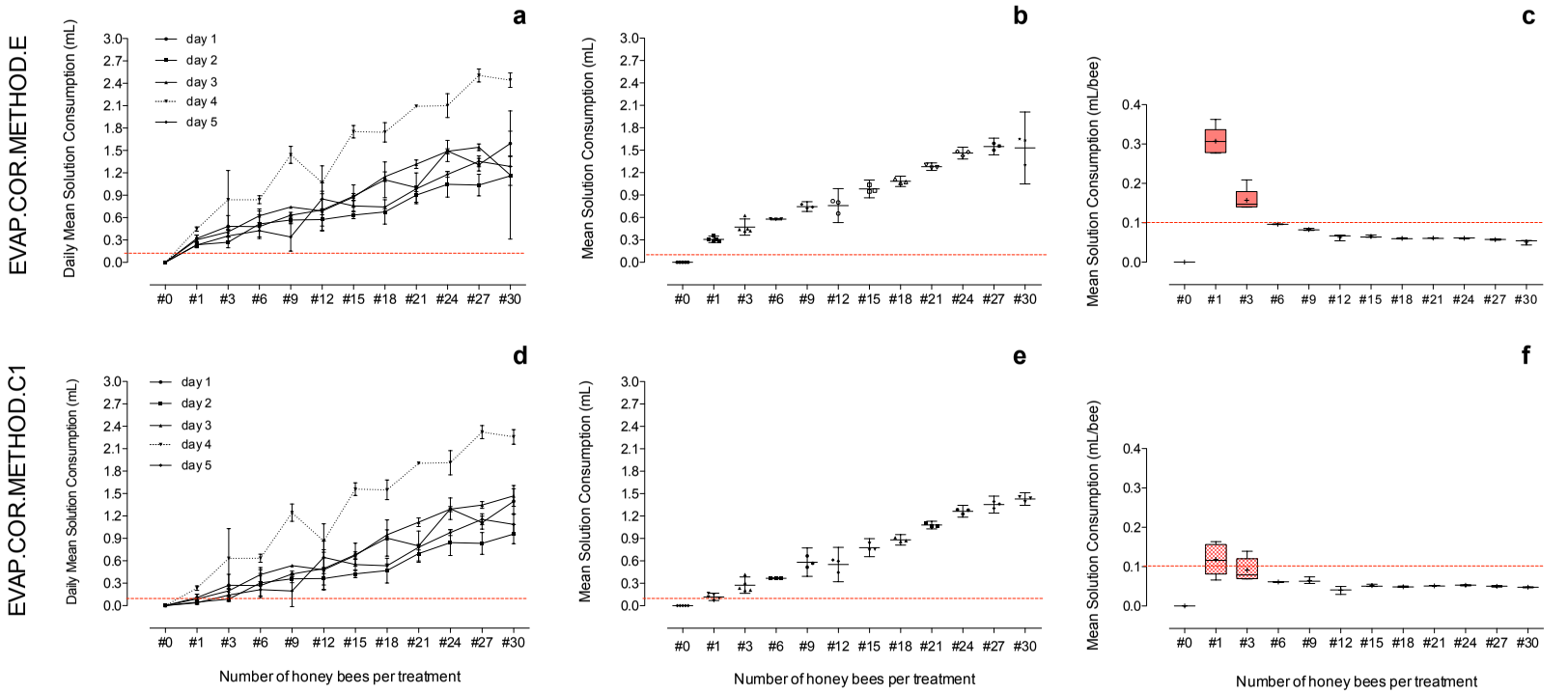

Figure 2.11 Testing the effects of the number of adult honey bees on sucrose consumption (1.0 M). Solution consumption was obtained by measuring the weight difference every 18—26h over 5 days. Treatment boxes varied in number of bees (#1—#30 bees/box). Treatments #1 and #3 had 1 and 3 bees/box, respectively, and comprised N= 5 box replicates; all other treatments included N= 3 box replicates. Treatment #0 was displayed only for visual reference. Consumption was corrected for evaporation losses. Density factor was employed (1.12 g/mL sucrose solution). Line plots **a)** and **d)** show the daily sucrose consumption (mL/box) per treatment (Mean±SD). Scatter plots **b**) and **e)** shows the total mean (—) consumption (mL/box) (±95% CI) for each treatment over 5 days. Panels **c)** and **f)** show sucrose volume consumption per treatment per bee. Box-plots indicate the minimum and maximum range of values for each independent treatment, (—) and (+) indicate median and mean, respectively. The horizontal dashed lines highlighted in red indicate the assumed readability threshold for the balance used (0.1mg=0.1mL= 100µL of water). Panels a, b and c) represent consumption measurements corrected for evaporation by subtracting the mean evaporation loss (%) correction (Δraw.consumption-Δevaporation/5days) - Method E (in Section 2.4 in this Chapter). Panels d, e and f) depict consumption corrected for evaporation by subtracting the mean solution loss (g) (Δraw.consumption-Δevaporation/5days) - Method C1 (in Section 2.4 in this Chapter). Here, negative consumption values produced by mass subtraction (3.85 %) were considered as missing values.

2.8.3 Study 5. Discussion

This study intended to identify potential factors that may compromise the reliability of consumption values in cases whereby measured responses occur near or below the limit thresholds of detection of the equipment. This may occur when the number of subjects in feeding boxes is small (< 6 bees/box). As predicted, measuring diet consumption in boxes containing less than 6 bees is likely to produce values below or near the detection threshold of the balance (i.e. 0.10 mg). Here, for all treatments except #1 and #3, total consumption of sucrose solution (1.0 M) resulted in 40–50 μL per young adult bee (maximum of 6 days old at the end of the experiment). This is in accordance with previously reported work whereby caged adult honey bees ingested on average 40–50 μL/bee (0–7 days old) or 150–180 μL/bee (> 21 days old) (Paoli *et al.*, 2014). By dividing the total consumption figure by the number of live bees in each box, I assumed that every bee within the box reached to food and ingested similar amounts. These results suggest that smaller cohorts of caged-bees (#1 and #3) are likely to ingest more solution in long-term feeding setups (> 48h feeding with food provided *ad libitum*) compared to larger cohorts of bees. If this is true, one could reason that in smaller groups, bees have straight access to food, don't need to spend time in grooming other bees or cage related activities and, in theory, could feed more often. Yet, by comparing total consumption/box (Figure 2.11 b, e) vs. total consumption/bee (Figure 2.11 c, f) for individual treatments, it is more likely that these measurements resulted from an artifact of the method. While Figures 2.11 (b, e) imply that consumption/box is near the lower limit threshold of dectection (0.10 mg) of the balance, Figure 2.11 (c, f) denote that measuring consumption/bee in boxes with less than 6 bees could overestimate how much food is consumed per bee (120–300 μL/bee in treatment#1). These measurements are not physically possible for individual honeybees, as crop volume varies from 30–60 μL. Therefore, I conclude that data obtained when there are very small cohorts of bees (i.e. < 6 bees/box) is not reliable using this method.

Until recently, few studies have addressed the reliability of consumption measurements and food distribution within group feeding in laboratory settings (e.g. caged-bees). Brodschneider et. al. cleverly added ^{14}C polyethylene glycol as a radioactive marker to sucrose solutions (50 % w/v) and assessed food distribution and consumption among individual bees within feeding cohorts (Brodschneider *et al.*, 2017). They found that caged adult bees do not share food equally, i.e. on average there was 8.8-fold difference in consumption between every two bees inside the box. They estimated this figure by dividing the highest intake/bee by the smallest intake/bee, thus obtaining the inner 80 % intake ratio. This does not support the assumption that each bee in a group consumes the same. The same study, reached the conclusion that a best approach to attain uniform food distribution within cage cohorts is using boxes harbouring 10 (instead of 30) newly emerged bees from the same brood comb (colony) (Brodschneider *et al.*, 2017). In contrast to my feeding box design, these authors used modified disposable plastic cups (200 mL) after (Evans *et al.*, 2009), and did not test feeding groups bearing less than 10 bees/box as I did here.

The current data, show that the total solution consumption between #12 and #15, and #30 is different (Figure 2.11 b and e). Yet, when presented as consumption/bee, treatments #12, #15 and #30 were not statistically different. This suggests that individual bees within these boxes consumed on average the same solution (Figure 2.11 c and f). Whether each single free-flying bee inside those boxes ingested the same volume of solution over 5 days cannot be deduced from my data. Feeding boxes with larger number of bees, e.g. > 15 bees/box seem more reliable to assess by measuring consumption by weight difference. Food could also be shared by trophallaxis (mouth to mouth transfer), indicating that some box-mates may ingest more solution than others (Brodschneider *et al.*, 2017), but more bees per box produce less variability in food consumption measurements per bee. In addition, the type of feeding box may also influence how bees reach food. Cages of 400 mL capacity, such as in this study, compared to 200 mL (Evans *et al.*, 2009; Brodschneider *et al.*, 2017), may warrant better access to food in groups of 30 bees.

The results presented here demonstrate that using different adjustment methods to correct for solution losses have a distinct impact, and possibly misleading ones, on the consumption measurements of small feeding cohorts (e.g. < 6 bees/box) (Figure 2.11 c and f). Against excepted, method E that deduces a normalised mass loss (%) instead of absolute mass loss (g) produced larger consumption artifacts in treatments #1 and #3. It seems unlikely that a single young worker ingested an average of ~300 μL on her own. Therefore, using a radioactive marker (e.g. ^{14}C PEG) to label solution fed to bee cohorts seems a better approach to produce more accurate and reliable consumption measurements, especially when single or small bee cohorts are tested. This procedure, however, can be expensive and is time-consuming. There are other laboratory protocols to test food consumption of single bees, which involve physically constraint (e.g. harness) (Rinder, 1976; Williams *et al.*, 2013). This method, if used, must be later supported by field or free-flying bees' data, as it may result in increased acceptance and consumption of unpalatable and harmful food (e.g. high salt) (Desmedt *et al.*, 2016).

Altogether, these results showed that *in vitro* caged experiments using worker honey bee cohorts are suitable to assess group feeding dynamics and consumption over extended periods. Assessing consumption as a measure of weight change of feeding tubes is only reliable if larger groups of bees per box are used (at least > 6 bees/box). Assuming that each bee in a box consumes the same amount of diet must depend on the cage design and the number of bees per box. Smaller cohorts may deliver misleading results and larger cohorts may reduce slightly the magnitude of measurements (Figure 2.11 c and f). In larger groups (e.g. 30 bees/box), some bees may ingest more than others and food sharing via trophallaxis is more likely to occur than direct contact with food. Yet, overall patterns of feeding and relative consumption between diet treatments are not expected to vary greatly. In any case, if uniform consumption within feeding groups is necessary, cohorts of 10–30 bees/box are desired. Similarly, the use of more accurate measurements (e.g. radioactive food labelling) are recommended (Brodschneider *et al.*, 2017). Furthermore, evaporation correction methods employed to adjust consumption results may also impact the

reliability of measurements in smaller feeding cohorts, and thus confound interpretation of results.

Based on these assessments, I propose two thresholds to help on deciding to accept or reject consumption measurements using current experimental set up and cohorts of N=30 bees/box. **Condition 1)** defined as the minimum number of live bees present in one unit box to be accepted as a reliable replicate of consumption. The minimum number of bees in one box at a certain time should be at least 20% of bees since day 1, but no less than 6 bees/box at a certain day (e.g. N= 30 bees/box from which 20% is N= 6 bees/box). **Condtion 2)** defined as the minimum number of reliable box replicates in one day (N= > 6 bees/box) to be accepted as a reliable daily consumption. The minimum number of reliable box replicates in a certain day should be 30% of the total number of box replicates (e.g. N= 10 boxes/treatment from which 30% is N= 3 boxes/day). In cases that consumption values did not reach these two assumptions, cut off thresholds must be applied and values removed from the datasets. Data obtained from feeding treatments likely to induce high bee mortality rates should be checked to respect these two assumptions. Applying these assumptions to process consumption measurements will provide more reliable measurements and increase confidence on results hereafter in this thesis.

2.9 Conclusion

In summary, preliminary no-choice assays (Study 1) even with low sample size of box units, but with sufficient bee subjects per treatment (N= 100–288 bees) served as ground work to explore feeding preferences and flagged high Cu and Ca diets detrimental for bee survival. Nuances involved in this experimental design and consumption measurements were further explored in this Chapter. I have alse dedicated some experiments to shelf position within incubators (Study 2), evaporation correction methodologies (Study 3), feeding tubes' position within boxes (Study 4), and finally the number of bees per feeding box. These experiments pointed to more effective practical approaches to conduct either feeding experiments and data processing. For example, I conclude that method E to adjust evaporation losses, side-by-side diet tubes with daily shift across the box for diet delivery, and feeding cohorts of 30 bees per box should be further employed for optimisation of the feeding experimental desings. Additionaly and most imporantly, two cut-off thresholds should be applied to process consumption data more reliably. The results presented in this Chapter led to the optimisation of the final experimental design used in feeding assays in Chapter 4.

3

GUSTATORY RESPONSES TO MINERAL SOLUTIONS BY INDIVIDUALLY HARNESSED FORAGER BEES

Chapter 3

Gustatory Responses to Mineral Solutions by Individually Harnessed Forager Bees

3.1 Highlights

- Proboscis responses to salts/metals in water were very weak and similar to water alone regardless concentration.
- Iron-enriched sucrose solutions elicited higher responses upon antennal stimulation, but not other minerals.
- Proboscis responses to Na, K, Zn and Mn in sucrose solutions (1.0 M) did not differ from control solutions (sucrose alone), but high Ca (2%), Fe (~0.6%) and Cu (~0.6%) seem to deter bees from feeding.

3.2 Abstract

Salts and metals are inorganic micronutrients necessary for basic metabolism. Besides its nutritional importance, salt in food can traditionally ascribe opposite hedonic values to food depending on its concentration. Studies from both mammals and insects revealed that low salt is attractive, but high salt deters feeding. Honey bees seem to detect and forage for salts from multiple sources, especially when presented in water (as electrolytes). Like other insects, bees respond to salts by means of gustatory sensilla located at the tip of the antennae, proboscis and tarsi. Floral nectar and pollen are prime food sources for bees and contain mineral salts. Yet, whether they detect salts and metals specifically in organic matrices (e.g. nectar, pollen) is still uncertain. Also, in contrast to salts, much less is known about the appetite effects of metals in either water or nectar-like solutions. Here, to assess whether gustatory sensilla on the antennae or proboscis (mouthparts) were sensitive to the presence of salts or metals in different dietary contexts, I tested bees' antennae with increasing concentrations of salts or metals in either water or sucrose solutions to elicit the Proboscis Extension Reflex (PER). Then, I assessed the drinking responses to mineral solutions by measuring whether bees were likely to consume a droplet of the same testing solution. Sodium (Na) is particularly important for phytophagous insects and is the main culprit underlying specific salt hungers. So, I next measured whether taste responses to Na were affected by a sucrose gradient (masking effect). Using this experimental approach, results indicated that both salts and metals in water at low concentrations (< 5 mM) are phagostimulatory to forager bees. Also, the bees' antennae were more responsive to accept K and Mn in water. In contrast, drinking assays showed that bees were more selective and less responsive to high levels of minerals in solution. This corroborates the importance of gustatory cues on the mouthparts to prevent ingesting noxious solutions (e.g. high metals). Unexpectedly, I did not find a masking effect of sucrose gradient on the perception of Na in solution as I was unable to discern whether Na was detected or not even at

high concentrations. These data together showed that individual bees can detect not only salts but also metal nutrients in water and nectar-like solutions to different extents within the same range of concentrations. The aim of this Chapter was to test whether bees, by means of gustation, respond to a range of relevant minerals in two dietary contexts (water or nectar-like solution). These results added baseline information to further assess the preingestive behaviour and regulation of mineral intake by adult bees in Chapter 4, Feeding Assays.

3.3 Background

Salts and metals are micronutrients naturally occurring in bee food (nectar/pollen/water), and fundamental for osmoregulation, neuronal function and metabolic reactions (Dow, 2017). These essential nutrients must, therefore, be acquired at low to moderate doses from food. When ingested in excess, salts/metals may induce dehydration or death by toxicity. The balance between sufficient or excessive doses is yet to be clarified in bees. Mineral requirements and associated feeding behaviours are not as well-understood for honey bees as for other insect pollinators (e.g. Lepidoptera) (Smedley and Eisner, 1996; Inoue *et al.*, 2012). However, different bee species have been found foraging in mineral-rich sources (e.g. brackish water, animal sweat, urine, tears) (Bänziger *et al.*, 2009; Abrol *et al.*, 2012). To my knowledge, Butler was the first to address seemingly salt preferences by water foragers (Butler, 1940). Later, von Frisch observed that dilute sucrose solutions (0.5 M) spiked with > 7.5 mM NaCl deterred bees from feeding, noting a low tolerance for 'saltiness'(von Frisch, 1934, 1967). Whitehead and Larsen have tested and described salt detection by different gustatory organs in honey bees (Whitehead and Larsen, 1976a; Whitehead, 1978). Since then, few studies have tackled salt perception and related behaviours in bees. Similarly, high mineral nectar contents also seem to deter bees from feeding (Waller, Carpenter and Ziehl, 1972; Afik, Dag and Shafir, 2008; Afik et al., 2014). Recently, one study addressed honey bee preferences elicited by

major electrolytes (Bonoan *et al.*, 2016). Yet, the extent of honey bee sensitivities to mineral salts and related detection mechanisms are still poorly understood.

Insects can perceive and learn the chemical composition of food by contact chemoreception (Marshall, 1934; von Frisch, 1934; Dethier, 1955; Whitehead and Larsen, 1976a; Whitehead, 1978). Taste is a sensory modality for evaluating the edibility of potential food sources and regulating ingestion and, therefore, taste can directly relate to insects' nutritional needs. Seminal studies in the blowfly, moths and bees established that hair-like cuticular extensions (taste sensilla) scattered across strategic body parts (e.g. antennae, proboscis, abdomen, tarsi) are the gateway for peripheral chemical detection and the initiation of feeding in most insects (Minnich, 1932; von Frisch, 1934; Dethier, 1955; Hodgson, 1957; Whitehead and Larsen, 1976). At the cellular level, single gustatory sensilla are typically innervated by 4–5 gustatory receptor neurons (GRNs) that can respond to multiple taste qualities. These GRNs can often respond to either attractive (e.g. sugar and low salt) or aversive (e.g. bitter and high salt) chemical stimuli by inducing or suppressing feeding behaviours; see reviews in fruit fly (Liman, Zhang and Montell, 2014), and honey bees (De Brito Sanchez, 2011). In contrast to olfaction and vision, gustatory responses in bees have only recently gained further attention . In adult worker honey bees, gustatory chaetica sensilla are mostly found on the antennal tips, mouthparts (galea, labial palps, glossa) and forelegs (tarsi) (Marshall, 1934; Whitehead and Larsen, 1976b; Mitchell, Itagaki and Rivet, 1999) (see Figure 1.3, Chapter 1 for an illustration of the worker honey bee head and proboscis).

Bees´exploratory gustation of rewards is perhaps accomplished mostly through the antennae or the tarsi while grooming and in hive (Whitehead and Larsen, 1976a; Whitehead, 1978). Whether bees detect specific salts by means of taste sensilla is still controversial. Recent studies, suggested that honey bee tarsi are likely to be tuned to perceive salts in water (De Brito Sanchez *et al.*, 2014). So far, electrophysiological and behavioural studies suggested that the sensitivity of taste sensilla are tuned to either sweet or bitter compounds. This is, in part, because sugars are predominant in floral nectars and biologically relevant as the major source of

food/energy for adult worker bees (for review see (Brodschneider and Crailsheim, 2010)). Whereas bitter compounds are typically toxic and avoided by insects (Meunier *et al.*, 2003; Hiroi *et al.*, 2004; Wright *et al.*, 2010; Muth, Francis and Leonard, 2016).

The molecular basis of peripheral taste in insects is mostly understood from *Drosophila* studies. The activation of GRNs within taste sensilla upon contact with dietary chemicals seems to occur via ligand-gated transmembrane proteins either directly (ion channel receptors, IRs) (Rytz, Croset and Benton, 2013; Zhang, Ni and Montell, 2013) or indirectly (G-coupled protein receptors, GRs) (Hiroi *et al.*, 2004; Montell, 2009). So far, the honey bee genome suggests a repertoire of 12 putative GRs and 21 IRs genes (Robertson and Wanner, 2006; Smith et al., 2011; Sadd et al., 2015), of which GRs appear to be expressed primarily in peripheral gustatory organs such as the antennae, galea, labial palps and legs (Simcock, Wakeling, *et al.*, 2017) with three appointed as sugar receptors, AmGr1 and AmGr2 (Jung *et al.*, 2015), and AmGr3 (Takada *et al.*, 2018). Though, nothing is yet confirmed for the remaining candidates nor to other taste modalities such as salt. Both mammals and insects seem to exhibit two distinct taste pathways for salt sensing associated with the hedonic value of food. These sensing mechanisms modulate feeding decisions and subsequent behaviour. The appetitive pathway is tuned for low salt detection, whereas the aversive pathway prevents ingestion of high-salt food. These findings demonstrate the importance of salt detection at appropriate concentrations. In *Drosophila*, two ENaC channel members (*ppk11* and *ppk19*) expressed in the terminal organ seem to be required to detect low salt in larvae (Liu et al., 2003). In adult flies, responses to low and high salt is mediated by two types of salt-responsive GRNs (Zhang, Ni and Montell, 2013). Low salt sensing in adults appears to require IR76b, from the ionotropic glutamate receptor family (Rytz, Croset and Benton, 2013). As for high salt sensing, it has been reported that two genes expressed in gustatory neurons in the terminal organ of larvae (*ppk19* and *sano*) are both required for high-salt sensing and avoidance behaviour (Alves et al., 2014).

Compared to salts, the effect of metals on feeding responses is less studied, but if consumed in excess, metals can be toxic and impair health. Few studies have tackled the toxicity of individual metals in bees, but some insects were reported to develop learned food aversions to high metals in food and to internally regulate metal toxicity (Stone, Jepson and Laskowski, 2002; Behmer *et al.*, 2005; Freeman *et al.*, 2007; Grześ, 2009; Green, Diaz and Tibbett, 2010; Russell *et al.*, 2011; Hurst, Stevenson and Wright, 2014; Bednarska and Stępień, 2015; Stolpe and Muller, 2016).

The primary focus of behavioural studies in honey bee gustatory perception has mostly explored how bees detect and accept sweet tastants. This is accomplished by triggering an appetitive reflex (PER) by touching gustatory organs (antennae, proboscis, tarsi) with sugar solutions (Haupt, 2004; De Brito Sanchez *et al.*, 2005). Other tastants, such as bitter compounds, do not elicit PER per se, but gustatory reponses can still be indirectly assess by absence of PER (Wright *et al.*, 2010, 2013; Cocco and Glendinning, 2012). So far, gustation of minerals has been only reported in a handful of studies (Hladun *et al.*, 2012; De Brito Sanchez *et al.*, 2014; Lau and Nieh, 2016), some of which addressed the behavioural preferences for minerals of water forager bees (Butler, 1940; Bonoan *et al.*, 2016; Dorian and Bonoan, 2016; Lau and Nieh, 2016). Yet, there is a lack of understanding on the gustatory responses of honey bees to dietary metals in water or floral rewards. Honey bees require both salts and metals in their diet. It is, therefore, important to investigate their gustatory responses to minerals especially in low and ecologically relevant concentrations (see Tables 1.3 and 1.4, Chapter 1). Salt taste modality often relates to Na, and could be either a positive or negative gustatory stimulus, therefore, I aimed to ascertain whether gustatory sensilla on the antennae or proboscis (mouthparts) of forager honey bees were sensitive to the presence of salts (NaCl, KCl, $CaCl_2$, $MgCl_2$) or metals ($FeCl_2$, $ZnCl_2$, $CuCl_2$, $MnCl_2$) in two dietary contexts (water or 1.0 M sucrose). Simultaneously, I assessed the masking effect of a sucrose gradient in the phagostimulatory power of Na. I expect that salts and metals can be detected in a concentration-dependent fashion, with high concentrations triggering rejection, but low concentrations eliciting acceptance. By testing salts or metals at the same range

of concentrations I predicted that salt or metal taste identity can influence overall gustatory responses (appetitve/aversion).

3.4 Methods

In this section, I present the methods and methodologies used to conduct behavioural gustatory assays on individual harnessed forager honey bees.

3.4.1 Animal Stocks

Honey bee colonies were kept at the Newcastle University campus (rooftop) apiary between May to October. Experimental bees arrived from at least two out of five colonies of *Apis mellifera* hybrid (Buckfast honey bee stocks, England) or *Apis mellifera carnica* (Carniolan honey bee stocks, Slovenia). Subsets of forager bees specialize in water, pollen or nectar collection, which can be assessed by the extent bees respond to sucrose solutions (Page, Erber and Fondrk, 1998), for review see (Scheiner, Page and Erber, 2004). Sucrose responsiveness predicts foraging specificity (pollen/nectar or water collection) and serves as a proxy to estimate the physiological state of the colony (Pankiw and Page, 2000). Pollen foragers tend to be more selective and respond to lower concentrations of sucrose (high PER), while nectar foragers tend to respond mostly to high sucrose concentrations (Page, Erber and Fondrk, 1998). At the level of the proboscis (the last interface prior ingestion), buckfast bees were more attracted to lower sucrose levels (S6 Figure, Appendix A). Therefore, I reasoned that carniolan bees were mostly nectar foragers. No feeding supplement was given to these colonies during experimental period. *Apis mellifera* is not a protected species, therefore no ethical permission was necessary for this study. Carniolan stocks kept at our campus (rooftop) apiary were chosen for gustatory assays described in this chapter.

3.4.2 Animal Collection and Harnessing

Forager honey bees from carniolan stocks (*Apis melifera carnica*) of mixed-age (> 21 days old) (Winston, 1991) were collected for gustatory assays. A wire mesh was placed at the entrance of each hive for a maximum 3 h. Bees returning to the hive on taking off the mesh were caught in glass vials (20 mL, disposable scintillation vials); up to three bees/vial (Figure 3.1). Ventilated glass vials were preferred to cold anesthethize bees as it cools down faster and reduces stress (Human *et al.*, 2013). Collection was directed in a timely manner. Each round of collection took ~30 min to avoid bee starvation before moving them to lab conditions. Each round of 30 min collection rendered up to 300 bees (3x100 vials). Even numbers of bees were collected from each colony, time and weather permitting. Collection periods took place beetween May and September (2015 - 2016), from 9 am to 2 pm or 1 pm to 5 pm. At the end of each feeding experiment, bees remaining alive were frozen-killed. They were not returned to the hive because 1) animals were pooled together from different colonies; 2) they could contaminate nest mates with high mineral food via trophalaxis (food transfer mouth to mouth) (Crailsheim, 1998). at the entrance of the hive upon return. After collection, bees were individually harnessed as described in the following sub-section, and as shown in Figures 3.1 and 3.2.

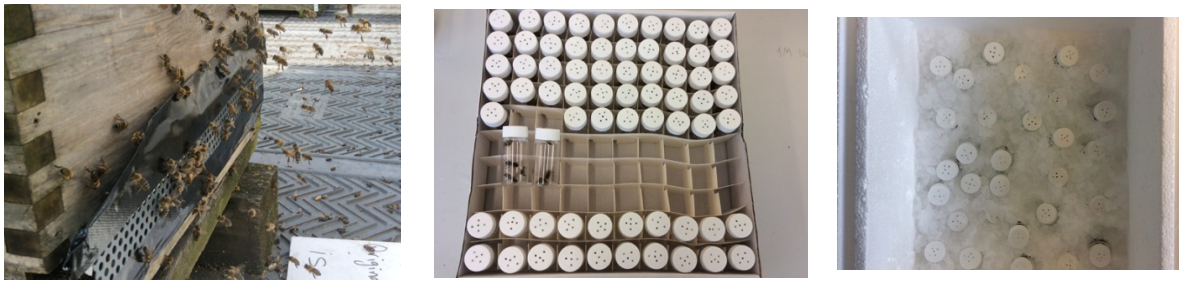

Figure 3.1 Animal collection. From left to right, forager bees were collected in ventilated glass vials (up to 3 bees/vial), and later anesthetized in a bed of ice until become motionless (up to 3 min). Then forager bees were harnessed and assigned to experimental treatments.

3.4.3 Animal Preparation and Harnessing for Taste Assays

After collection, bee vials were placed on ice (3 – 5 min) for cold-induced immobilisation. Individual bees were then strapped into small harnesses (metal or modified-plastic pipette tips) with a strip of labelling tape (Figure 3.2). Each bee was placed in a harness covering the abdomen and thorax, while leaving the neck and the forelegs free. This allowed the animal to freely move the head and groom herself if needed. Also, this method is less prone to physically damage the animal or trap accidentally the unfolded proboscis. To prevent starvation and standardise experimental animals prior testing, bees were fed 4 μL of 1.0 M sucrose solution. Bees that did not drink the droplet at this point were removed from the study. Bees were then left alone to adapt to the harness inside ventilated plastic recipients in the dark for at least 1 h. To prevent over-starvation and standardise motivation in experimental animals, bees were then fed 4 μL of 1.0 M sucrose solution and allowed to adapt for 1 h prior testing. Bee motivation to elicit PER was mildly and negatively affected with 1 h starvation time compared to 2 or 3 h. However, bees were equally motivated to feed after 1 or 3 h, and therefore 1 h starvation time was selected for further studies (see S7 Figure, Appendix A).

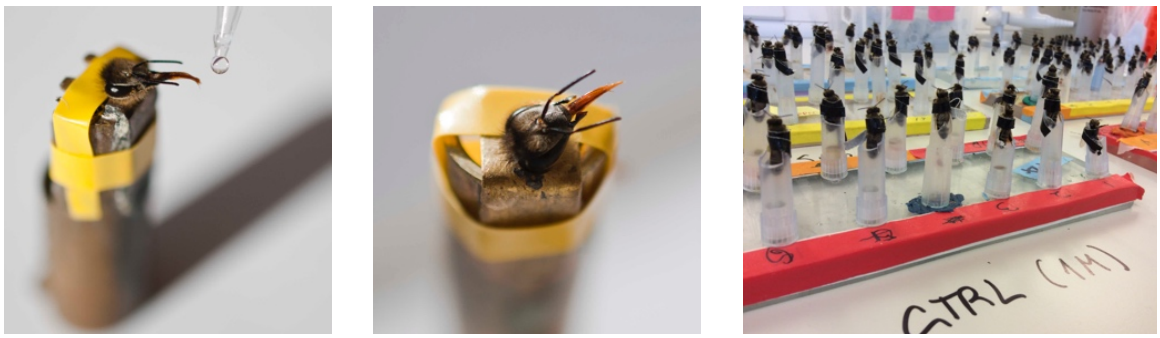

Figure 3.2 Preparation of individual honey bee for gustatory behavioural assays. After anesthetization by cold, individual bees were strapped into small harnesses (3 cm length) (metal or modified-plastic pipette tips) with a strip of labelling tape. Here, bees on the left were mounted in metal harnesses. Yet, due to number constraints, assays were mostly conducted using plastic-modified tips as depicted in the far-right image.

3.4.4 Antennal Stimulation – PER responses

Individually-harnessed honey bees were stimulated by gently touching one antenna with a toothpick soaked in the testing solution and its PER recorded (Figure 3.3 a). Positive PER is considered the full extension of the proboscis for at least 3 s, otherwise is recorded as a negative response or absent PER. Each responsive bee was tested with the full range of mineral treatment solutions. Responses were recorded as a binary variable; whether PER was elicited (PER= 1) or not (PER= 0). Stimulation started with control solution (sucrose or distilled water) and followed by ascending concentrations of single salts (NaCl, KCl, $CaCl_2$ or $MgCl_2$ at 5, 50, 500 mM) or metals ($FeCl_2$, $ZnCl_2$, $CuCl_2$ or $MnCl_2$ at 1, 10, 100 mM) in eihter distilled water (water group) or 1.0 M sucrose (sucrose group) to elicit PER (Table 3.1). The interval between each treatment was 4–5 min. Each stimulation with testing soution was preceded by stimulation with water alone to test for motivation and control for increased sensitization or habituation to repeated simulation, especially within the sucrose group (Page, Erber and Fondrk, 1998; Scheiner, 2004; Haupt and Klemt, 2005). The water group comprised N= 109–120 and N= 115–219 bees/treatment for salts and metals, respectivelly. The sucrose group included N= 34–113 and N= 35–200 bees/treatment for salts and metals, respectivelly. To test whether anion type has an effect on gustatory responses, bees were stimulated with increasing concentrations of

two metals (Fe and Cu) in either water or sucrose 1.0 M. Fe was provided as chloride (inorganic salt, $FeCl_2$) or ammonium citrate (organic salt, FAC). Cu was used as either chloride ($CuCl_2$) or sulfate ($CuSO_4$) salts (both inroganic forms). The stimuli concentrations and procedures were performed as described above. Only animals that survived until the last treatment solution was tested were considered for further analysis.

3.4.5 Proboscis Stimulation – Drinking Responses

Antennal assays, which did not involve feeding, were not expected to alter bees' motivational state. Therefore, the total number of bees was randomly split and assigned to different groups to test the proboscis gustatory responses to salts or metals. Each group represented one treatment solution and included ~ 20 bees per round. After antennal PER, bees rested for 10 min before commencing proboscis stimulation – drinking assays (Figure 3.3 b). To assess motivation and gustatory responses on the proboscis, individuals were stimulated on the antenna with a toothpick soaked in 1.0 M sucrose solution and the antennal PER response was recorded (as previously described). If the bee responded positively to sucrose, a droplet (4 μL, micropipette) of the testing solution was then delivered to the tip of the extended proboscis and the bee allowed to drink. If PER was not elicited, the bee was removed from the experiment. Drinking was defined as when the droplet decreased in size when the proboscis was extended and contacted the droplet. Responses were measured as a binary variable depending on whether the bee drank the whole droplet of the testing solution. A positive drinking response was considered when the bee accepted and drank the whole droplet. Each bee was assessed for one treatment solution only. Single salts and metals were independently tested in either water or sucrose solutions (Table 3.1). The water group included N= 20–145 and N= 35–145 bees/treatment for salts and metals, respectively. The sucrose group included N= 35–50 and N= 28–40 bees/treatment for salts and metals, respectively. Only measurements from bees responding to sucrose antennal

stimulation were plotted. To test whether anion type would influence the gustatory responses, bees were stimulated with increasing concentrations of two metals (Fe and Cu) in two forms, $FeCl_2$ vs. FAC or $CuCl_2$ vs. $CuSO_4$. The stimuli concentrations and procedures were performed as described above. Only animals that survived until the last treatment solution was tested were considered for further analysis.

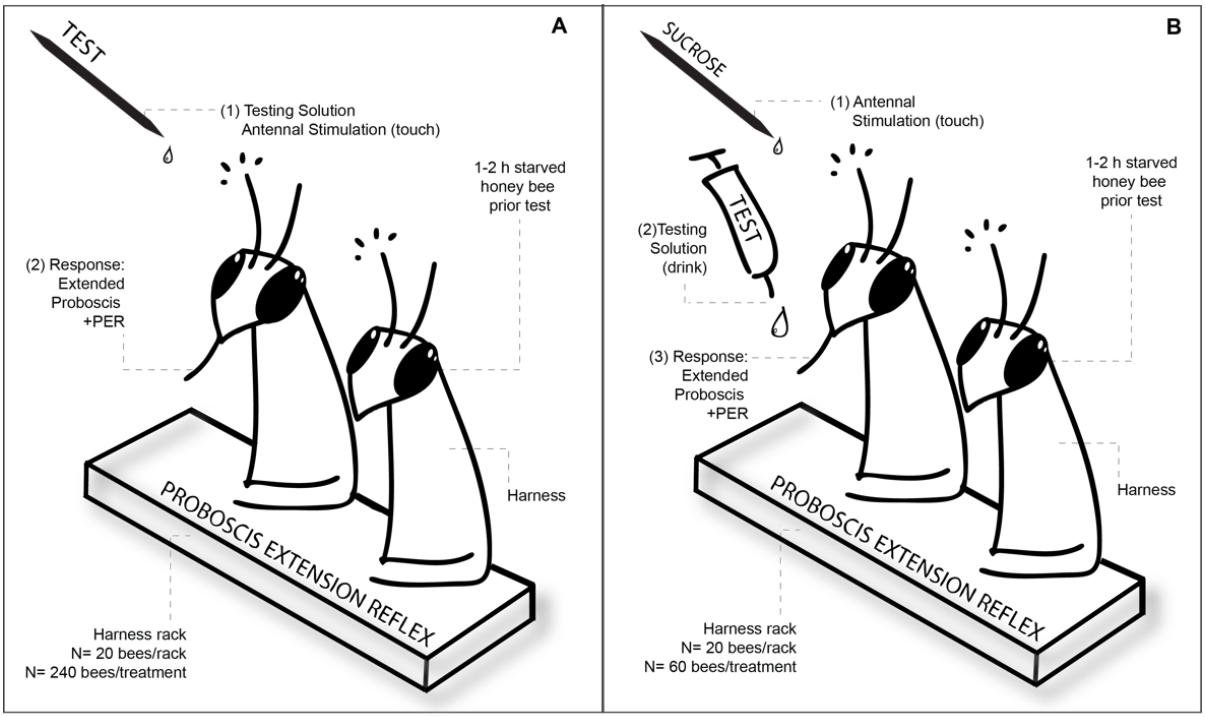

Figure 3.3 Gustatory Behavioural Assays. **a)** Antennal stimulation (PER – Proboscis Extension Reflex). Harnessed honey bees were stimulated on the antennae with testing solutions (no feeding). **b)** Proboscis stimulation – drinking response. Bees were first stimulated on the antennae to assess motivation using sucrose (1.0 M) solution. If PER was elicited, the proboscis was stimulated by contact with a droplet (4 μL) of the testing solution and allowing it to drink. If PER was not elicited, the bee was withdrawn from the assay. If PER was elicited, but no further attempt to drink the droplet of solution occurred, the drinking response was recorded as negative.

3.4.6 Effect of Sucrose Gradient on the Gustatory Perception of Sodium Solutions

To test whether and how nectar-like solutions varying in sucrose concentration impact gustatory responses to Na, I performed both antennal (PER) and drinking assays as previously described. Here, I tested a concentration series of Na (0, 1, 10, 100, 1000, 1,000, 22,989 ppm) dissolved in one of five sucrose background

solutions (0 (water), 0.1, 0.25, 0.5 and 1.0 M) (Table 3.2). Each group (sucrose solution varying in Na concentration) was tested indeptendently. Antennal assays comprised N= 348–360 bees/treatment, and proboscis assays included N= 104–120 bees/treatment. Only animals that survived until the last treatment solution was tested were considered for further analysis.

3.4.7 *Solution Stimulus*

Liquid solutions were prepared as previously described. Two groups of stimuli were defined by background solution: water or sucrose groups. To test responses to mineral solutions, distilled water or sucrose solutions (1.0 M) were supplemented with a single mineral at three levels of concentration (salts: 5, 50, 50 mM; metals: 1, 10, 100 mM) (see Table 3.1). Solution concentrations were calculated using the formula: Mass(g) = [Molar ($molL^{-1}$)] x Molecular Weight Salt ($gmol^{-1}$) x Final Volume Solution (L). Solutions prepared were aimed for ppm units, but were calculated in molar units by distraction. This realisation arose after some animals have been tested and already sacrificed, so I decided not to abort this experiment and to continue. Nonetheless, this was an opportunity to test not as much appetitive stimulus, but towards aversive instead. This range of concentrations were estimated in molar concentration, though they still fit within the ppm range tested in feeding assays in Chapter 4. The range of concentrations was drawn after (Herbert and Shimanuki, 1978) and contents found in bee pollen (see Table 1.4, Chapter 1).Exploratory range-finding studies conducted in Chapter 2 confirmed these concentrations suitable. Hereafter, a colour code was attributed to each salt/metal to ease contextualization of the graphics when necessary. As such, **sucrose control** diets are shown in blank, **sodium (Na)** diets are depicted in grey, **potassium (K)** in yellow, **calcium (Ca)** in brown, **magnesium (Mg)** in red, **iron (Fe)** in orange, **copper (Cu)** in aqua blue, **zinc (Zn)** in green and **manganese (Mn)** in magenta hues. Bees are expected to respond in a bimodal manner: extend its proboscis (mouthparts) in expectation of food reward or not. PER responses to sucrose alone were taken as

positive control when assessing individual motivation. Each solution was used fresh and at room temperature prior testing. Treatment solutions were measured independently, one trial each. For each testing group, water or sucrose alone were considered control treatments (no minerals added).

Table 3.1. Concentrations of mineral salt solutions for gustatory assays. All minerals tested were chloride salts. Concentrations presented respect cation.

Salts				**Metals**			
Stimulus	**mM**	**ppm**	**%**	**Stimulus**	**mM**	**ppm**	**%**
Sodium	0	**0**	0	Iron	0	0	0
	5	115.	0.01		1	55.9	0.01
	50	1,150.	0.12		10	559.	0.06
	500	11,495.	1.15		100	5,585.	0.60
Potassium	0	0	0	Copper	0	0	0
	5	196.	0.02		1	63.6	0.01
	50	1,955.	0.20		10	636.	0.07
	500	19,549.	1.95		100	6,355.	0.65
Calcium	0	0	0	Zinc	0	0	0
	5	200.	0.02		1	65.4	0.01
	50	2,004.	0.20		10	654.	0.06
	500	20,039.	2.00		100	6,539.	0.60
Magnesium	0	0	0	Manganese	0	0	0
	5	122.	0.01		1	55.0	0.01
	50	1215.	0.12		10	550.	0.06
	500	12,152.	1.22		100	5,494.	0.06

Atomic mass of cations: 22.99 (Na^{+}), 39.10 (K^{+}), 40.08 (Ca^{2+}), 24.31 (Mg^{2+}), 55.85 (Fe^{3+}), 65.39 (Zn^{2+}), 54.94 (Mn^{2+}) and 63,55 (Cu^{2+}).

Table 3.2. Concentrations of Na solutions used to test the effect of a sucrose gradient on forager bee gustatory responses.

Stimulus	mM	ppm
Sodium (Na^+)	0	0
	0.04	1
	0.44	10
	4.35	100
	43.5	1000
	435.	10,000
	1000.	22,989

3.4.8 Statistical Analysis

Data was analysed using IBM SPSS Statistics for Macintosh (version 24.0, 2017). Factorial modelling was performed. Non-significant higher order interaction terms were removed to improve model fit. To analyse the average gustatory responses to testing solutions, I used Generalized Linear Models (GzLM) fitted with a binary logistic regression. In cases that models could not fit due homogeneous responses, either all positive (PER= 1) or negative (PER= 0), a single value was artificially changed within the data set to fit the binary logistic model. No further adjustments were made. Data was plotted using the original data.

Antennal and drinking assays were analysed independently. For each assay, three predictors were introduced in the model to estimate the effects on the response variable: background solution (water or sucrose), mineral type (salt or metal) and stimulus concentration (control, low, medium and high). Data from salt or metal treatment groups were analysed independently. Water and sucrose (1.0 M) alone were considered the control solutions. Therefore, and for each test, the independent variables were background solution (water/sucrose), mineral type (salt/metal) and stimulus concentration. For antennal assays (PER), Generalized Estimating Equations (GEE) models for repeated measures (within-subjects: stimulus concentration) were used to analyse the mean probability of bees responding to testing solutions upon

antennal stimulation. Individual bees were stimulated multiple times. For the drinking assays, GzLM (no repeated measures) were employed to analyse the mean probability of bees willing to consume the whole droplet of testing solutions.

For the effect of the anion, Fe and Cu were also analysed independently. The independent variables were background solution (water/sucrose), anion type and concentration.

For Na-only solutions in a sucrose gradient, background solution and Na concentrations were the independent variables. The response variable for all the assays was measured as a binary behavioural output.

3.5 Results

To identify whether forager honey bees are innately sensitive to mineral nutrients, I tested antennal PER and drinking responses of individual forager bees to a series of either salts or metals selected according to their importance and prevalence in bee nutrition. To ascertain the phagostumulatory strength of salts/metals in different dietary contexts, salts/metals were delivered in either water or sucrose (1.0 M). Salts and metals were analysed independently and, therefore, results are indicated separately. For both antennal and proboscis responses, results for salts are shown in Figure 3.4 and Table 3.3 and for metals in Figure 3.5 and Table 3.4.

3.5.1 Antennal Gustatory Responses to Salts in Solution

The salt-elicited antennal responses depended on the background solution, salt identity and salt concentration (Figure 3.4 a, b and Table 3.3). The average PER response was greatest for bees responding to K solutions (Figure 3.4 a). Surprisingly, when presented to the antennae bees were more attracted to K and Ca in water compared to Na (and Mg) (pairwise Seq. Bonf.: K vs. Ca, $P<0.01$, 95% CI [-0.26, -0.04]; Na, $P<0.001$, 95% CI [-0.39, -0.16]; Mg, $P<0.001$, 95% CI [-0.39, -0.17]; then Ca vs. K, $P<0.001$, 95% CI [0.04, 0.26]; Na, $P<0.001$, 95% CI [-0.23, -0.03]; Mg, $P<0.001$, 95% CI [-0.24, -0.03]). Na and Mg did not differ from one another across stimuli concentration. However, when bees were stimulated with the same salt stimulus but presented in sucrose-enriched solutions, PER responses did not follow the same pattern (Figure 3.4 b). In fact, the average response of antennal stimulation with salt-enriched sucrose solutions were similar to control solution (sucrose alone), regardless salt identity or concentration (> 90% PER responses).

3.5.2 Antennal Gustatory Responses to Metals in Solution

As for the metal group, the antennal stimulus-response function also depended on the background solution, metal identity and stimulus concentration (Figure 3.5 a, b and Table 3.4). Across all concentrations, PER responses of bees stimulated with metals in water increased compared to water control alone. Though further concentration-dependent increase was not observed (Figure 3.5 a). The antennae were most responsive to Mn in water solution as > 60% bees elicited PER at low Mn on average (pairwise Seq. Bonf.: Mn vs. Zn, $P<0.001$, 95% CI [-0.23, 0.04]; Fe, $P<0.001$, 95% CI [-0.37, -0.17]; Cu, $P<0.001$, 95% CI [-0.47, -0.31]). In contrast, bees rejected Cu solutions the most (less PER elicited) (pairwise Seq. Bonf.: Cu vs. Fe, $P<0.001$, 95% CI [0.06, 0.17]; Zn, $P<0.001$, 95% CI [0.17, 0.35]; Mn, $P<0.001$, 95% CI [0.31, 0.47]).

When bees were stimulated with metals on the antennae but in sucrose solutions instead, high Fe and Cu solutions massively decreased PER responses (Figure 3.5 b). Compared to Cu, Zn and Mn, the average greatest response was elicited by Fe stimuli (pairwise Seq. Bonf.: Fe vs. Mn, $P<0.001$, 95% CI [-0.24, -0.09]; Cu, $P<0.001$, 95% CI [-0.25, -0.04]; Zn, $P<0.001$, 95% CI [-0.29, -0.10]). Zn and Mn produced similar antennal responses. Bees seem to perceive the presence of low Cu even in sucrose-rich solution as there was a steep decrease in PER responses from control to low Cu (Figure 3.5 b). Overar all salts and metals, Cu appears to be mildly inhibitory. All stimuli were presented in ascending concentrations from no salt/metal to high salt/metal. Overall, when both salts or metals in water were presented to the bees' antennae, PER responses increased at low mineral levels, though responses did not increase/decrease significantly with increasing concentrations. In fact, bees responded equally well to the solutions, regardless of the concentration showing a flat gradient (Figure 3.4 a and 3.5 a).

3.5.3 *Effect of the Anion Type on the Antennal PER Responses to Metal Solutions*

Testing the anion effect on antennal PER responses of individual bees revealed that Fe treatments the background solution, anion type and metal concentration affected metal detection (GEE: soln x anion x conc: $\chi 2= 18.7$, $P< 0.001$; soln x anion: $\chi 2= 27.3$, $P<0.001$; soln x conc: $\chi 2= 51.2$, $P<0.001$; anion x conc: $\chi 2= 37.9$, $P<0.001$; soln: $\chi 2= 267.$, $P<0.001$; anion: $\chi 2= 0.64$, $P=0.64$; conc: $\chi 2= 48.7$, $P<0.001$). Likewise, for Cu treatments the background solution, anion type and metal concentration affected responses to Cu solutions (antennal stimulation. GEE: soln x anion x conc: $\chi 2= 12.0$, $P= 0.01$; soln x anion: $\chi 2= 5.19$, $P=0.02$; soln x conc: $\chi 2= 69.5$, $P<0.001$; anion x conc: $\chi 2= 25.5$, $P<0.001$; soln: $\chi 2= 382.$, $P<0.001$; anion: $\chi 22= 35.1$, $P<0.001$; conc: $\chi 2= 13.8$, $P=0.003$). For example, FAC (iron citrate) (vs. $FeCl_3$) and $CuSO_4$ (vs. $CuCl_2$) were more phagotilmulatory (> PER responses vs. control) when presented in water solutions (Fe: Figure 3.6 a, c; Cu: Figure 3.7 a, c). However, when presented in 1.0 M sucrose, and in contrast to their chloride forms, FAC and $CuSO_4$ elicited similar responses to sucrose alone across solution concentrations (Fe: Figure 3.6 b, d; Cu: Figure 3.7 b, d).

3.5.4 *Drinking Responses to Salts in Solution*

Upon proboscis stimulation, drinking responses of bees to both salts and metalsin water solutions were less pronnounced (Figure 3.4 c and 3.5 c). Almost none or less than 20% were likely to consume a droplet of any mineral solution after willingly responding to sucrose antennal stimulation. Results presented here only show data from bees motivated to drink, which scored positive PER to 1.0 M sucrose solution (a pre-requisite to preform the drinking assay; see methods section above). The likelihood of individual bees consuming salt water solutions depended on the background solution and stimulus concentration, but not salt identity (Figure 3.4 c; Table 3.3). No salt at any concentration was sufficiently attractive to be ingested by bees compared to distilled water alone. However, whenever these salts were

delivered with sucrose , nearly all bees were willing to ingest solutions presented to the proboscis regardless. While this was the case for Na, K and Mg, high Ca clearly deterred bees from feeding as demonstrated by the steep decline in average consumption (- 40% bees) (Figure 3.4 d). High Mg seemed to induce a slight decrease in bee responses, though it was not significant. Therefore, bees were less likely to consume high Ca-laced sucrose solutions compared to Mg, Na and K (pairwise Seq. Bonf.: Ca vs. Mg, P>0.05, 95% CI [-0.01, 0.17]; Na, P<0.001, 95% CI [0.05, 0.21]; K, P<0.001, 95% CI [0.03, 0.20]). None of the bees consumed the droplet of water alone.

Table 3.3 Generalized linear models[1] for gustatory responsiveness to salt solutions by antennal or proboscis stimulation as shown in Figure 3.4.

Source: Salts	**Wald $\chi 2$**	**df**	**P value**
Antenna			
(Intercept)	124.	1	**<0.001**
solution (soln)	307.	1	**<0.001**
salt	20.6	3	**<0.001**
concentration (conc)	25.7	3	**<0.001**
soln x salt	20.9	3	**<0.001**
soln x conc	31.4	3	**<0.001**
salt x conc	31.1	9	**<0.001**
Proboscis			
(Intercept)	0.83	1	0.36
solution (soln)	404.	1	**<0.001**
salt	7.07	3	0.07
concentration (conc)	1.76	3	0.62
soln x salt	1.75	3	0.63
soln x conc	20.4	3	**<0.001**
salt x conc	13.7	9	0.14

[1]GEE, repeated measures (within-subjects: conc) analysed the mean probability of bees eliciting PER to each testing solution upon antennal stimulation; GzLM (no repeated measures) analysed the mean probability of bees consuming the whole droplet (4 μL) of each solution upon proboscis stimulation; Values in bold highlight a probability value (P value) < 0.05, indicating a mean significant difference at the level of 5%. Non-significant higher order interactions were removed from the model in a stepwise manner.

Figure 3.4 **Gustatory responsiveness and proboscis extension reflex of forager honey bees by application to either the antennae or the proboscis of increasing salt concentrations in two background solutions (water or sucrose).**

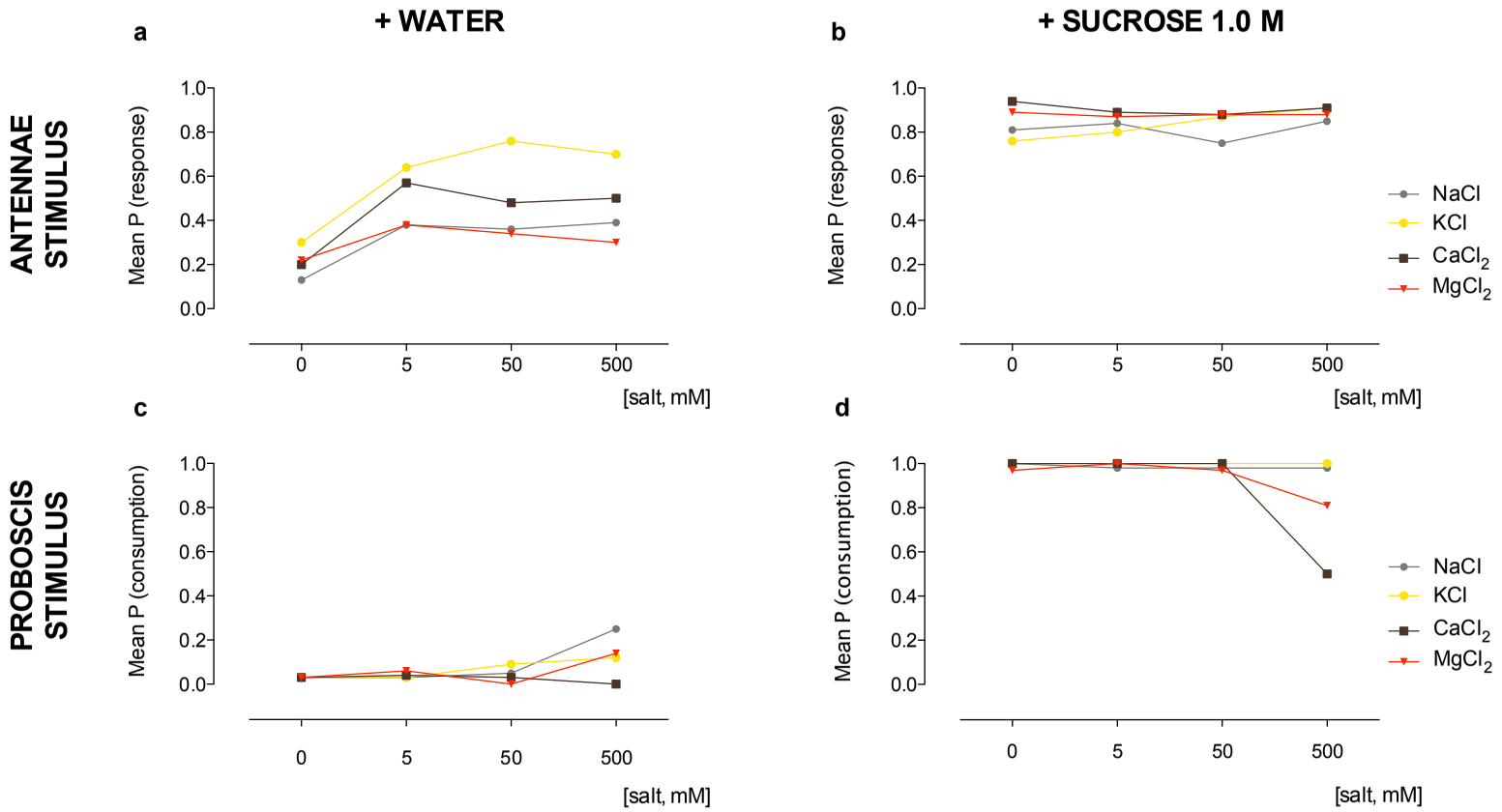

Figure 3.4. Proboscis extension reflex (PER) and drinking responses after stimulation on the antennae (a: in water, N=109-120/treatment; b: in sucrose 1.0 M, N=34-113/treatment) or the proboscis (c: in water, N=20-145/treatment; d: in sucrose 1.0 M, N=35-50/treatment), respectively. Stimuli consisted of increasing concentrations of salts (Na, K, Ca and Mg; 0 (control), 5, 50 and 500 mM in distilled water or sucrose (1.0 M) solutions. In panels a and b are shown the mean probability of bees eliciting PER to each solution upon antennal stimulation. Panels c and d show honey bee drinking responses as the mean probability of bees that consumed the whole droplet (4 µL) of each solution.

3.5.5 Drinking Responses to Metals in Solution

Drinking responses to metal solutions depended on both metal identity and concentration, but not on the background solution being either water or sucrose (Figure 3.5, Table 3.4). This suggests that taste sensilla on the proboscis of bees are more sensitive to perceive variations on metal nutrient composition in food on inducing appropriate feeding responses.

Table 3.4 Generalized linear models[1] for gustatory responsiveness to metal solutions by antennal or proboscis stimulation as shown in Figure 3.5.

Source: Metals	**Wald χ^2**	**df**	**P value**
Antenna			
(Intercept)	0.001	1	0.98
solution (soln)	467.	1	**<0.001**
metal	42.2	3	**<0.001**
concentration (conc)	71.7	3	**<0.001**
soln x metal	144.	3	**<0.001**
soln x conc	109.	3	**<0.001**
metal x conc	55.9	9	**<0.001**
soln x metal x conc	58.4	9	**<0.001**
Proboscis			
(Intercept)	10.5	1	**<0.001**
solution (soln)	390.	1	**<0.001**
metal	32.5	3	**<0.001**
concentration (conc)	32.2	3	**<0.001**
soln x metal	2.85	3	0.42
soln x conc	7.39	3	0.06
metal x conc	25.1	9	**<0.001**

[1]GEE, repeated measures (within-subjects: conc) analysed the mean probability of bees eliciting PER to each testing solution upon antennal stimulation; GzLM (no repeated measures) analysed the mean probability of bees consuming the whole droplet (4 μL) of each solution upon proboscis stimulation; Values in bold highlight a probability value (P value) < 0.05, indicating a mean significant difference at the level of 5%. Non-significant higher order interactions were removed from the model in a stepwise manner.

Similar to salt stimuli, metals in water delivered to the tip of the proboscis did not induce consumption as responses did not differ from distilled water alone across all concentrations (Figure 3.5 c). Across all metal treatments, < 10% on average consumed a droplet of water. In contrast, metal-enriched sucrose solutions were likely to be accepted by individual bees, with as much as > 80% bees consumed Fe, Mn, Zn and Cu solutions at low and medium levels (Figure 3.5 d). Even though solutions (< 100 mM) were readily consumed, compared to Fe, Mn and Zn, Cu induced a slight decrease in responses. Zn and Mn stimuli yielded both antennal PER and drinking responses similar to sucrose control . In contrast, high Fe and Cu stimuli were not accepted by bees, since the average response values decreased significantly relative to the control. This suggests that Fe and Cu are repulsive even when paired with sucrose in solution (1.0 M). Sucrose taste did not mask the presence of neither high Fe nor Cu (100 mM) (Figure 3.5 d). In general, bee responses to high Fe and Cu contrasted those elicited by similar concentrations of both Zn and Mn (pairwise Seq. Bonf.: Cu vs. Mn, $P<0.001$, 95% CI [0.60, 1.04]; Zn, $P<0.001$, 95% CI [0.58, 1.05]; Fe, $P>0.05$, 95% CI [-0.06, 0.17]; and Fe vs. Mn, $P<0.001$, 95% CI [0.67, 1.07]; Zn, $P<0.001$, 95% CI [0.65, 1.07]; Cu, $P>0.05$, 95% CI [-0.17, 0.06]). In sum, salt and metal stimuli in either water or sucrose solutions appear to induce similar proboscis responses in general. It is also worth noting that taste sensilla on both the antenna and the mouthparts may have different sensitivities to metals, especially at high concentrations (100 mM, ~0.65%) (Figure 3.4 b, d and 3.5 b, d).

Figure 3.5 Gustatory responsiveness and proboscis extension reflex of forager honey bees by application to either the antennae or the proboscis of increasing metal concentrations in two background solutions (water or sucrose).

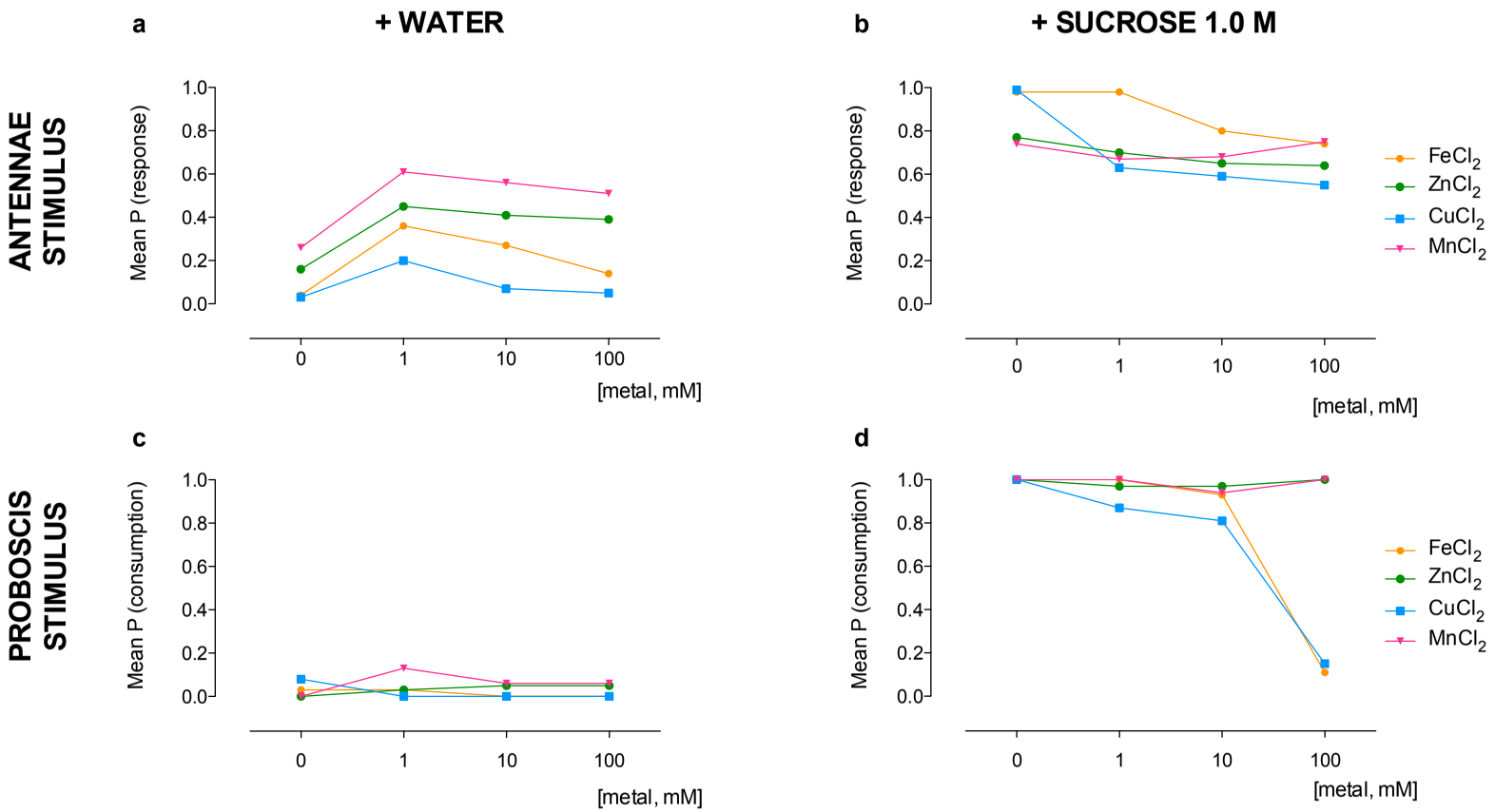

Figure 3.5. Proboscis extension reflex (PER) and drinking responses after stimulation on the antennae (a: in water, N=115-219/treatment; b: in sucrose 1.0 M, N=35-200/treatment) or the proboscis (c:: in water, N=35-145/treatment; d: in sucrose 1.0 M, N=28-40/treatment), respectively. Stimuli consisted of increasing concentrations of metals (Fe, Zn, Cu and Mn; 0 (control), 1, 10 and 100 mM in distilled water or sucrose (1.0 M) solutions. In panels a and b are shown the mean probability of bees eliciting PER to each solution upon antennal stimulation. Panels c and d show honey bee drinking responses as the mean probability of bees that consumed the whole droplet (4 μL) of each solution.

3.5.6 *Effect of theAnion Type on the Drinking Responses to Metal Solutions*

The anion effect on the likelihood of bees consuming Fe solutions revealed that drinking responses were a function of background solution, anion type and metal concentration (GzLM: soln x anion x conc: $\chi 2$= 8.35, P= 0.04; soln x anion: $\chi 2$= 0.32, P=0.58; soln x conc: $\chi 2$= 7.72, P=0.05; anion x conc: $\chi 2$= 7.36, P= 0.06; soln: $\chi 2$= 171., P<0.001; anion: $\chi 2$= 7.87, P=0.01; conc: $\chi 2$= 8.74, P=0.03). As for Cu stimuli, drinking responses significantly depended on solution and anion type and solution and concentration (GzLM: soln x anion x conc: $\chi 2$= 3.12, P= 0.37; soln x anion: $\chi 2$= 0.68, P=0.41; soln x conc: $\chi 2$= 10.4, P=0.02; anion x conc: $\chi 2$= 10.7, P= 0.01; soln: $\chi 2$= 172., P<0.001; anion: $\chi 2$= 23.1, P<0.001; conc: $\chi 2$= 3.56, P=0.31). Overall, bees accepted better FAC (vs. $FeCl_3$) and $CuSO_4$ (vs. $CuCl_2$) when presented in water solutions, but almost 2x less in magnitude compared to the antennal responses (Fe: Figure 3.6 c, d; Cu Figure 3.7 c, d). Also, when in sucrose solutions, chloride forms were better detected at the proboscis level, with high concentrations rejected. On the contrary, high FAC or $CuSO_4$ were not rejected; solution droplets were still consumed at similar extents as control solution (sucrose alone).

Figure 3.6 **Effect of anion type on proboscis extension reflex and drinking responses of forager honey bees by application to either the antennae or the proboscis of increasing iron concentrations in two background solutions (water or sucrose).**

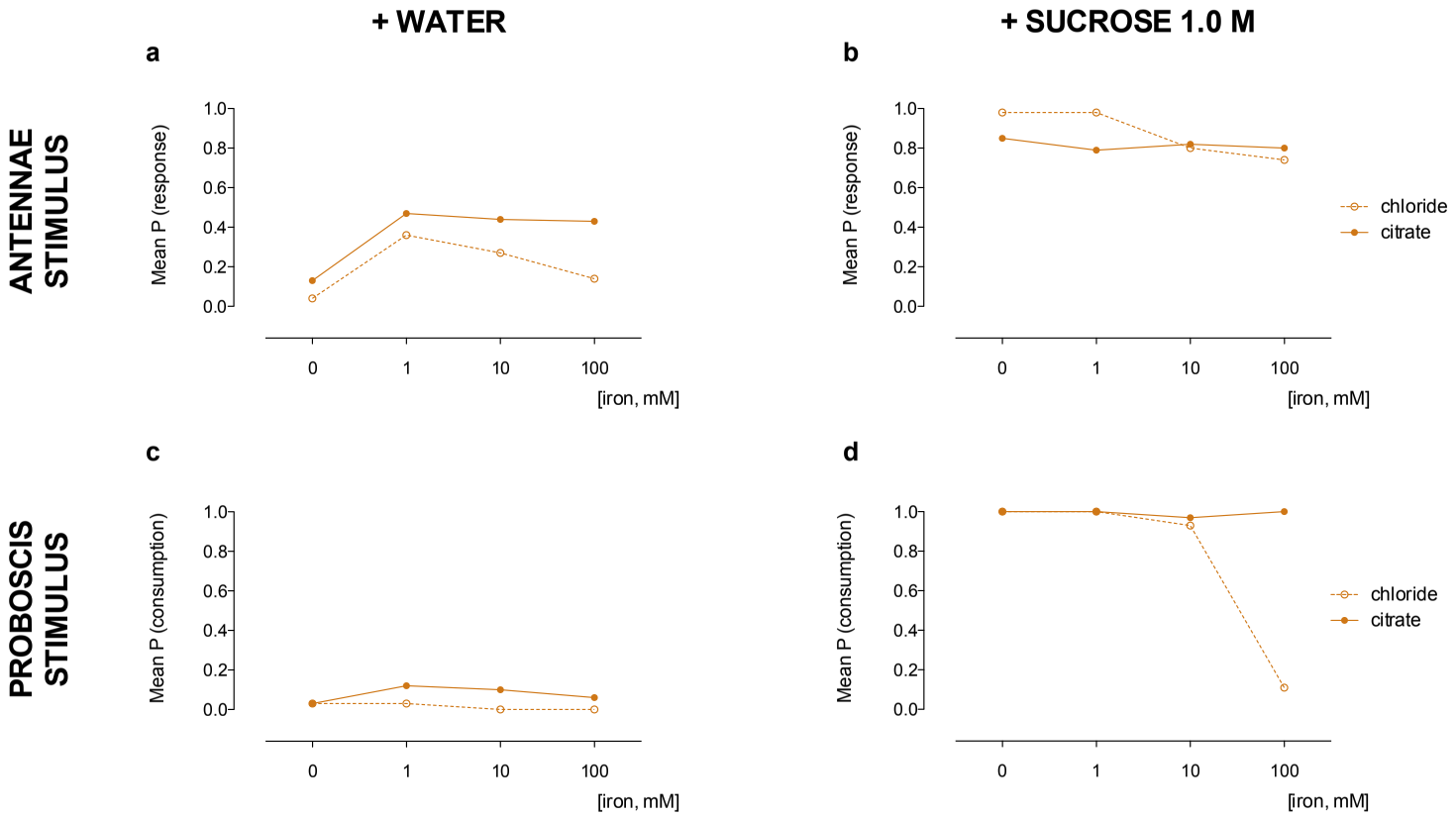

Figure 3.6. Proboscis extension reflex (PER) and drinking responses after stimulation on the antennae (a: in water, N=101-119/treatment; b: in sucrose 1.0 M, N=104-200/ treatment) or the proboscis (c:: in water, N=35-145/treatment; d: in sucrose 1.0 M, N=29-40/treatment), respectively. Stimuli consisted of increasing concentrations of iron chloride or citrate; (0 (control) - 100mM) in distilled water or sucrose (1.0 M) solutions. In panels a and b are shown the mean probability of bees eliciting PER to each solution upon antennal stimulation. Panels c and d show honey bee drinking responses as the mean probability of bees that consumed the whole droplet (4 μL) of each solution.

Figure 3.7 **Effect of anion type on proboscis extension reflex and drinking responses of forager honey bees by application to either the antennae or the proboscis of increasing copper concentrations in two background solutions (water or sucrose).**

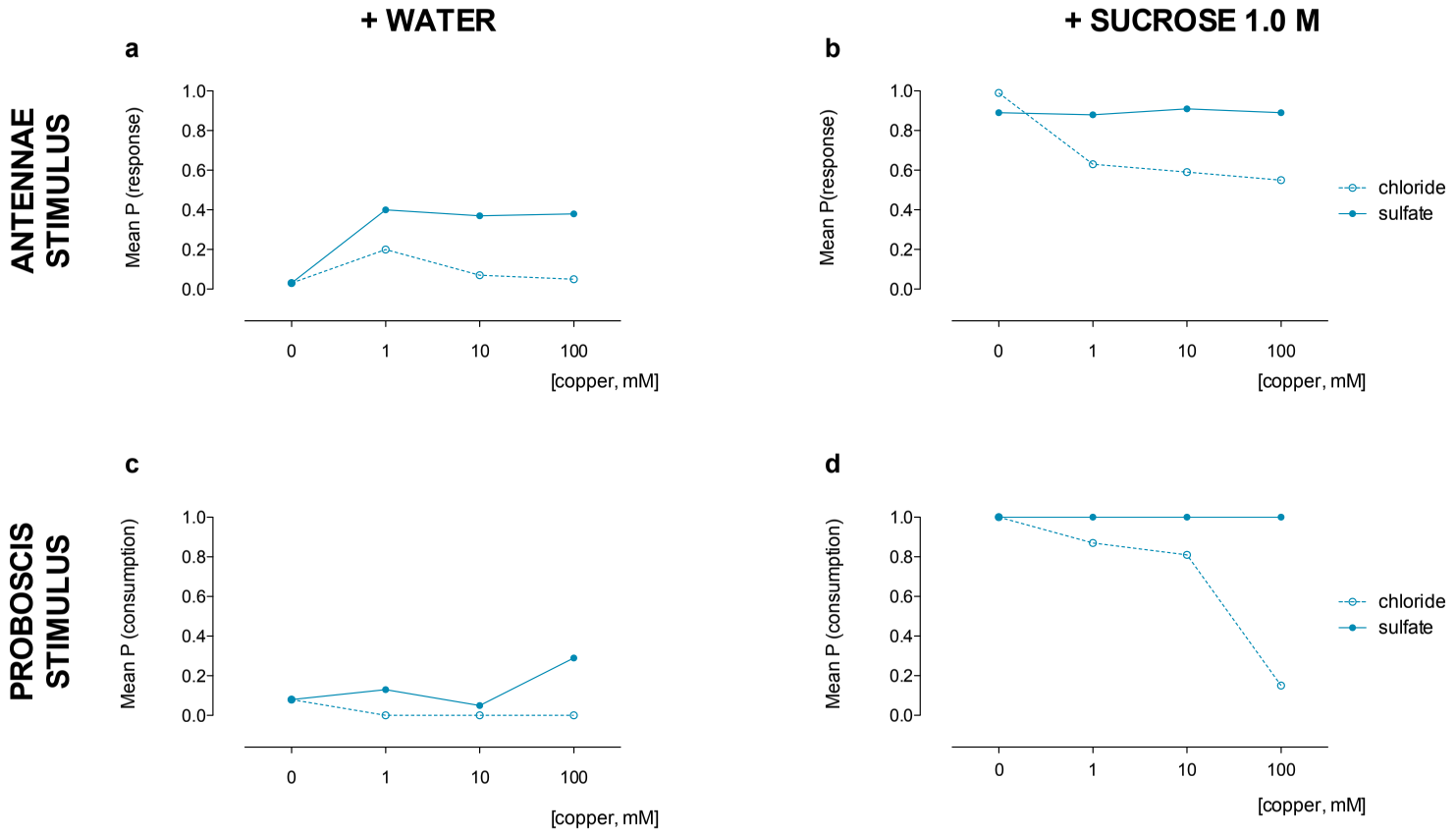

Figure 3.7. Proboscis extension reflex (PER) and drinking responses after stimulation on the antennae (a: in water, N=117-219/treatment; b: in sucrose 1.0 M, N=37-199/ treatment) or the proboscis (c:: in water, N=37-145/treatment; d: in sucrose 1.0 M, N=35-40/treatment), respectively. Stimuli consisted of increasing concentrations of copper chloride or sulfate; (0 (control) - 100mM) in distilled water or sucrose (1.0 M) solutions. In panels a and b are shown the mean probability of bees eliciting PER to each solution upon antennal stimulation. Panels c and d show honey bee drinking responses as the mean probability of bees that consumed the whole droplet (4 μL) of each solution.

3.5.7 Effect of a Sucrose Gradient on the Antennal PER responses to Na concentrations

To understand further how sucrose concentrations impact the gustatory perception of salts in bees, I also tested increasing concentrations of Na in a sucrose gradient. PER responses depended on both Na stimulus concentration and sucrose gradient (background solution) (Figure 3.8, Table 3.5). However, current data were not conclusive on the masking effect of sucrose in the detection of Na in solution. Bees from different sucrose treatments (0, 0.1, 0.25, 0.5 and 1.0 M) responded positively (> 90%) regardless of Na concentration.

As for the water group, Na slightly increased the phagostimulatory effect of solutions as PER increased when compared to water alone. Na was added to water as low as 1 ppm Na^+ (0.04 mM; 0.0001%) (Figure 3.8 a). The average value of PER responses was greatest for 0.25 M sucrose treatment (~ 99%), and similar at all levels of Na and the control solution (0.25 M sucrose only) (overall results for 0.25 M sucrose, X_2: 2.75, df=6, P= 0.84; then 1.0 M sucrose, X_2: 21.0, df=6, P<0.01; then 0.1 M sucrose, X_2: 30.2, df=6, P<0.01; then 0.5 M sucrose, X_2: 125., df=6, P<0.01; then water, X_2: 1409., df=6, P<0.01).

Table 3.5. Generalized linear models[1] for gustatory responsiveness to increasing concentrations of Na in a sucrose gradient by either antennal (PER) or proboscis (consumption) stimulation as shown in Figure 3.8.

Source	Wald $\chi 2$	df	P value
Antenna			
(Intercept)	1976.	1	**<0.001**
solution (soln)	225.	4	**<0.001**
concentration (conc)	153.	6	**<0.001**
soln x conc	236.	24	**<0.001**
Proboscis			
(Intercept)	192.	1	**<0.001**
solution (soln)	907.	4	**<0.001**
concentration (conc)	24.6	5	**<0.001**
soln x conc	222.	20	**<0.001**

[1]GEE, repeated measures (within-subjects: conc) analysed the mean probability of bees eliciting PER to each testing solution upon antennal stimulation; GzLM (no repeated measures) analysed the mean probability of bees consuming the whole droplet (4 μL) of each solution upon proboscis stimulation; Values in bold highlight a probability value (P value) < 0.05, indicating a mean significant difference at the level of 5%. Non-significant higher order interactions were removed from the model in a stepwise manner.

3.5.8 Effect of a Sucrose Gradient on the Drinking responses to Na concentrations

The mouthparts of bees detected Na in solution, but were less responsive to Na stimuli when associated with lower sucrose concentrations. Two clear response groups could be identified (Figure 3.8 b). Bees offered Na in water or 0.1 M sucrose were much less likely to drink the whole droplet of solution. In contrast, bees delivered Na in either 0.25 M, 0.5 M or 1.0 M sucrose readily accepted and consumed the droplet solution even at very high Na (22,989 ppm Na^+; 1.0 M; 2.3%). The average consumption response was highest for 0.5 M sucrose (97%), then 0.25 M sucrose (95%), then 1.0 M sucrose (89%), then water (21%) and 0.1 M sucrose (18%). Almost 80% of bees from the water group accepted the droplet of distilled water (control). With Na in solution, however, consumption responses decreased to average values similar to previous experiments (Figure 3.4 c). Pairwise comparisons revealed the overall responses to Na concentrations were no different between water and 0.1 M sucrose treatments (pairwise Seq. Bonf.: water vs. 0.1 M sucrose, P=0.99, 95% CI [-0.04, 0-05]; 1.0 M sucrose, P<0.001, 95% CI [0.75, 0.87]; 0.25 M sucrose, P<0.001, 95% CI [0.77, 0.87]; 0.5 M sucrose, P<0.001, 95% CI [0.78, 0.88]).

Figure 3.8 **Gustatory responsiveness and proboscis extension reflex of forager honey bees by application to either the antennae or the proboscis of increasing concentrations of Na solutions in sucrose gradient.**

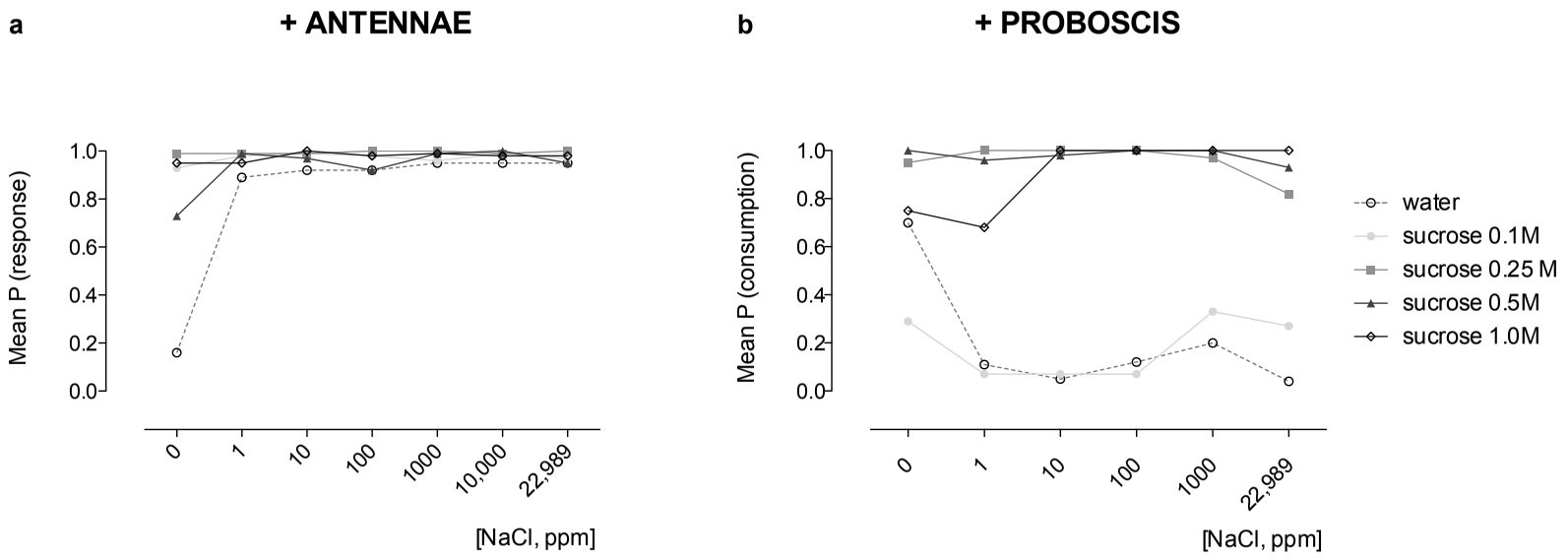

Figure 3.8. Proboscis extension reflex (PER) and drinking responses after stimulation on the antennae (a: N= 348-360) or the proboscis (b: N=104-120), respectively. Stimuli consisted of increasing concentrations of Na (0 (control) - 22,989 ppm) in distilled water, 0.1M, 0.25M, 0.5M and 1.0 M sucrose solutions. The testing solutions between assays were the same, except that 10,000 ppm of sodium was not tested in drinking assays. In panel a is shown the mean probability of bees eliciting PER to each solution upon antennal stimulation. Panel b shows honey bee responses to proboscis stimulation as the mean probability of bees that consumed the whole droplet (4 μL) of each solution.

3.6 Discussion

In this study I used the PER protocol, which is a commonly used laboratory assay to assess innate appetitive behaviours and gustatory perception in honey bees (Takeda, 1961; Bitterman *et al.*, 1983; Simcock, Gray, *et al.*, 2017). As such, my first aim tested how single salts or metals in solution affected taste responses of forager bees in two chemosensory organs (antennae or mouthparts). In honey bees, taste sensilla are found on the antennae, mouthparts and tarsi (Whitehead and Larsen, 1976a, 1976b; Whitehead, 1978; De Brito Sanchez *et al.*, 2014). Antennae seem mostly tuned to detect sugars (Haupt, 2004; De Brito Sanchez *et al.*, 2005), but also perceive salts (De Brito Sanchez *et al.*, 2005; Lau and Nieh, 2016). High salts and metals in food are known to deter insect feeding (Hagler, 1990; Boyd, 2009; Mogren and Trumble, 2010; Afik *et al.*, 2014; Stolpe and Muller, 2016). But in another study, Se solutions did not elicit PER, but were still ingested by honey bees (Hladun *et al.*, 2012), which suggest that certain metals may not be detected by contact chemoreception.

3.6.1 Antennal and Drinking Gustatory Responses to Mineral Solutions Differ in Sensitivity

Antennal gustatory responses to major salts in water have been previously investigated using PER techniques (De Brito Sanchez *et al.*, 2005; Lau and Nieh, 2016). De Brito Sanchez et al. (De Brito Sanchez *et al.*, 2005) suggested the existence of two responsive GRNs to 50 mM NaCl (1,150 ppm Na^+), but not to 10 mM KCl (391 ppm K^+) on the antennal taste sensilla. Lau and Nieh (Lau and Nieh, 2016) found maximum antennal PER to 1.5 % Na and Mg salts (~600 mM) compared to K and P in water, which, interestingly, were much higher than the average Na and Mg concentrations found in bee-collected fresh water (0.013 % or 5.7 mM Na^+; 0.003 % or 1.2 mM Mg^{2+}; 0.001% or 0.3 mM K^+). Here, I tested NaCl, KCl, $MgCl_2$ and $CaCl_2$ individually at 5.0, 50 and 500 mM (for all 0.01–2.0%). Antennal responses by restrained bees were maximum for K (50 mM; 1,955 ppm; 0.2% K^+), then Ca (5.0 mM;

200 ppm; 0.02% Ca^{2+}). Na and Mg solutions induced similar, but lower responses overal with maxium PER (~ 40 %) at 5.0 mM (115 ppm; 0.01% Na^{+} or 122 ppm; 0.01 % Mg^{2+}) (Figure 3.4 a). The lower and medium range of concentrations tested here can occur in bee-collected pollen (see Table 1.4, Chapter 1). Yet, the lowest concentrations seem more realistic in pollen, and the highest very unlikely to occur (with the exception of K, possibly) (see Table 1.4, Chapter 1).

Similar to (Lau and Nieh, 2016), antennal responses to salts in water were phagostimulatory at lower levels compared to water alone, but did not decrease at higher saline levels (10 % salt). This resulted in a seamingly flat response to high salt levels after initial increase (no salt to low salt). By stimulating the antennae of bees with increasing concentations of salts and possibly decreasing the phagostimulatory power, bees have adapted to the salt stimuli and responses were constant (Lau and Nieh, 2016). As proposed in (Lau and Nieh, 2016), this could be also the case of sensory adaptation, which is often described as a decrease in sensitivity due to continuos stimulation (e.g. serial antennal touch) and, thus, increase in detection/acceptance thresholds. Electrolyte concentrations as high as 500 mM (Figure 3.4 a, 3.5 b) or even 1.0 M (see Figure 3.8 a) still induced high PER responses. It should be also noted that my experiments and the one conducted by (Lau and Nieh, 2016) differ in two important aspects. First, I tested a mixture of forager bees collected from the hive entrance; they conducted PER on water foragers only. Foraging specialization (e.g. pollen, nectar, water) has long been associated to differences in antennal sucrose responsiveness (Pankiw and Page, 2000; Scheiner, Page and Erber, 2004), so it is possible that the water foragers have a different range of sensitivities to salts than the average forager population from a colony. Therefore, I speculate that gustatory responses to mineral salts may also be influenced by foraging specialization, pollen and water foragers may be less sensitive to mineral salts (i.e. higher thresholds). Second, (Lau and Nieh, 2016) tested PER responses to a salt concentration gradient (high to low). On the contrary, I followed the protocol often used to test sucrose solutions, with solute concentration increasing throughout the testing period (Scheiner, Page and Erber, 2004).

Contrasting to water solutions, single salts presented in 1.0 M sucrose scored all high PER responses regardless of salt and increase in concentration (Figure 3.4 b). The masking effect of high sucrose (Cocco and Glendinning, 2012) may in part explain the observed ceiling effect; for more about "the ceiling effect" see (Salkind, 2010). Alternatively, the internal state of each bee may have influenced gustatory responses to mineral solutions. The nutritional state of adult workers have been associated with differences in the expression of gustatory receptor genes in chemosensory organs tuned for sugar detection (Simcock, Wakeling, *et al.*, 2017; Takada *et al.*, 2018). Guiraud et al. tested the antennal gustatory capability of honey bees to discriminate between a range of solutions of different taste modalities. These authors recorded the occurrence of the Sting Extension Reflex (SER) (Núñez *et al.*, 1983) to gustatory stimuli by pairing a tastant with a mild electric shock (Guiraud *et al.*, 2018). They found that harnessed honey bees had poor antennal gustatory ability to distinguish either between NaCl vs. KCl (both at 100 mM), and 100 mM NaCl vs. 3.0 M (Guiraud *et al.*, 2018).

Taste sensilla on either the antennae or the mouthparts show different sensitivities to a range of tastants even within the same taste modality (e.g. sugars, salts) (De Brito Sanchez *et al.*, 2005; Simcock, Gray, *et al.*, 2017). Upon proboscis stimulation in drinking assays, bees did not respond more than they did to water alone (Figure 3.4 c and 3.5 c). But when minerals were added to sucrose solutions, only Fe (100 mM; 5,585 ppm) and Cu (100 mM; 6,355 ppm) induced clear aversion (Figure 3.4 d and 3.5 d). Data showed that taste organs in honey bees are likely to accommodate different behavioural taste responses depending on mineral salt identity, background solution (water or sucrose) and location of taste sensilla (antennae or mouthparts) (Figure 3.4 and 3.5). The floor effect observed in the drinking responses suggest a higher sensitivity for mineral detection at the level of the proboscis. This is reasonable to assume as the mouthparts constitute the last 'gateway' before ingestion. Regardless of the concentration, responses were similar to the water control, when electrolytes were presented to the mouthparts, < 20% bees were likely to consume the solution (Figure 3.4 c and 3.5 c). Though, bees elicited

PER to sucrose solutions (pre-requisite to assess motivation), the internal state of forager bees cannot be ruled out as a possible explanation to the poor acceptance observed for these solutions.

Salt responses of the gustatory neurons in the taste sensilla on the probosces of adult honey bees have been better characterized than on the antennae. Extracellular tip-recordings of chaetica sensilla displayed on the proboscis of nurse bees revealed, at least, one GRN responding to a range of electrolytes such as K^+, Li^+ and Na^+, which increased linearly to the log of cation concentration (Whitehead and Larsen, 1976a; Whitehead, 1978). In these studies, salt responses were characterized by lower firing rates and higher detection thresholds (200–300 mM) with higher sensitivity to KCl > NaCl (Whitehead and Larsen, 1976a; Whitehead, 1978). In contrast, the response of the gustatory sensilla on the proboscis to divalent metals (e.g. Mg^{2+}, Ca^{2+}) are less well understood. In blowflies (Evans and Mellon, 1962), butterflies (Inoue *et al.*, 2012), and honey bees (Whitehead and Larsen, 1976a; Whitehead, 1978) electrophysiological results for Mg^{2+} and Ca^{2+} most often produced erractic and small amplitude spikes compared to NaCl and KCl responses. I assessed the gustatory behavioural responses to divalent salts/metal solutions. I found that Ca elicited the second highest responses at the level of the antennae (Figure 3.4 a), but was deterrent at high levels when presented to bees' mouthparts (Figure 3.4 d). Other studies reported that Mg in water was phagostimulatory at 1.5 % (Lau and Nieh, 2016) and freely consumed at 1.0 % (Bonoan *et al.*, 2016). Present behavioural data show that bees respond to divalent metals and seem to be more sensitive to Fe and Cu, than to Zn and Mn at high concentrations (Figure 3.5 a, b, d). To the best of my knowledge, my data are the first to report appetitve gustatory responses in honey bees to salts at ecologically more relevant concentrations (see Table 1.4, Chapter 1), and, especially, to metal nutrients. Previously reported data focused on antennal gustatory responses of water forager to salts at levels higher levels (from ~300 ppm) (Bonoan *et al.*, 2016; Lau and Nieh, 2016). Others have assessed PER and drinking responses to Se solutions to assess its toxicity effects and impact on learning ability (Hladun *et al.*, 2012; Burden *et al.*, 2016). Here, I not only showed that bees have

divergent gustatory responses and sensitivity to different metals in solution, but also that the mineral form (i.e. conjugated anion) can also affect gustatory responses and acceptance to certin metals (Figures 3.4–3.7).

3.6.2 Gustatory responses to minerals are shaped by concentrations but not for all minerals

Next I aimed to assess whether background solution, i.e. distilled water or 1.0 M sucrose, influenced taste perception of salts or metals at three concentrations. Like other animals, bees need mineral salts and forage specifically for these nutrients in water sources (Butler, 1940; Abrol *et al.*, 2012; Bonoan *et al.*, 2016). While floral nectar does contain mineral salts, sucrose is the main carbohydrate and, thus, is an important stimulus for honey bees (Nicolson and Thornburg, 2007; Afik, Dag and Shafir, 2008; Afik *et al.*, 2014).

Salt-elicited gustatory responses are often shaped by concentration, such that low concentrations are phagostimulatory and high concentrations are aversive. From molecular and behavioural studies in *Drosophila* and mammals (e.g. mice), NaCl is attractive when animals taste low concentrations (<100 mM), but aversive when they contact high concentrations (500 mM) (Chandrashekar *et al.*, 2010; Zhang, Ni and Montell, 2013). Here, I expected similar responses in the adult worker honey bee. This is, acceptance and increasing number of positive responses at low mineral concentrations, followed by decreasing responses when concentrations were high. So far, high salt concentrations have been used in conditioning studies to induce aversive responses (> 3.0 M NaCl; 68,700 ppm; 6.9% Na^{+}) on either the antennae or mouthparts of honey bees (Abramson, 1986; Bhagavan and Smith, 1997; Wright, Choudhary and Bentley, 2009; Ayestaran, Giurfa and De Brito Sanchez, 2010; De Brito Sanchez *et al.*, 2015). Consistent with previoius behavioural and electrophysiological works (De Brito Sanchez *et al.*, 2005, 2014; Lau and Nieh, 2016), my data showed that honey bee workers seem to prefer dilute (< 50 mM for salts; < 1.0 mM for metals) over concentrated saline solutions (500 mM for salts; 100 mM

metals) (Figure 3.4 a, d and 3.5 a, d). Other studies combining both behvaioural and electrophysiological techniques showed that low NaCl; 10 mM or 150 mM was preferred in puddling butterflies (*Papilio* sp.) (Inoue *et al.*, 2012) and in blood-sucking insects (*Rhodnius prolixus*) (Pontes, Pereira and Barrozo, 2017), respectively. This suggest that between "low and high", there must be an optimum and preferred salt concentration, which suits each insect's biological needs. This dynamic resembles that of Bertrand's rule, which refers to increasing doses of a nutrient are beneficial until an optimum is reached; further increase at higher concentrations brings health disadvantages (Bertrand, 1912). However, bees did not show a clear bell-shaped dose-response curve, but rather one-step increase from control to low-salt/metal (Figure 3.4 a, 3.5 a) or one-step decrease at high/salt metal-laced sucrose solutions (Figure 3.4 d, 3.5 d).

Bees were more likely to detect mineral salts in water when in contact with their antennae, especially for K and Mn, which showed maximum PER responses (~60 %) (Figure 3.4 a, 3.5 a). It has been suggested that taste sensilla on the bees' mouthparts are more sensitive to a range of tastants and, thus, allow better discrimination and appropriate feeding decisions (Wright *et al.*, 2010). As refered above, minerals in water were not consumed more nor less than water (control) alone (floor effect); average responses did not differ from distilled water alone (< 20 %) (Figure 3.4 c, 3.5 c). However, when minerals were presented in sucrose solutions at low levels, bees were willing to consume those solutions (Figure 3.4 d, 3.5 d). Based on these results only, and because high concentrations of salts/metals (with the exceptions of Fe and Cu) did not decrease drinking responses, I cannot determine whether these minerals were truly accepted or were masked by 1.0 M sucrose as background solution.

Nectars containing high mineral contents (e.g. avocado, *Persea americana* or onion, *Allium cepa*) are less attractive to the European honey bees (Waller, Carpenter and Ziehl, 1972; Afik, Dag and Shafir, 2008; Afik et al., 2014). This feeding deterrence has mostly been attributed to high concentrations of K (> 3,500 ppm) and phosphate (> 600 ppm) ions, which naturally occur in avocado nectars (Afik, Dag and Shafir,

2008; Afik *et al.*, 2014), and high K in onion nectars (13,000 ppm; 332 mM; 1.3% K^+) (Hagler, 1990). Here, individually harnessed bees were not deterred by solutions containing K as high as 500 mM (19,549 ppm K^+) (Figure 3.4 b, d). Other minerals such as Se (1.5 ppm) did not deter flower visitation (Hladun *et al.*, 2013). Here, in contrast to freely moving forager bees, individually harnessed bees seem to accept these solutions and showed lack of aversion (Ayestaran, Giurfa and De Brito Sanchez, 2010; Desmedt *et al.*, 2016). Unlike other taste modalities (e.g. bitter), high Na in sucrose solutions (e.g. 3.0 M NaCl), even in harnessed bees, are rejected (Desmedt *et al.*, 2016). Such concentrations, however, are very unlikely to occur in floral nectars, which usually contain low levels of Na (< 10 mM; 230 ppm; 0.02% Na^+) (Nicolson and Worswick, 1990; Adler, 2000; Afik et al., 2006). These results showed that > 80% bees responded with antennal PER or by consuming a droplet of 500 mM Na-containing sucrose solutions (Figure 3.4 b, d). Only a mixture of 1.0 M sucrose and 500 mM $CaCl_2$, 100 mM $FeCl_2$ or $CuCl_2$ appeared to be sufficiently distasteful to deter consumption (Figure 3.4 d, 3.5 d).

So far, the current work is the first to assess taste responses of honey bees to relevant metal nutrients such as Fe, Zn, Mn and Cu. These metals can often be regarded as environmental pollutants when ocuring in high levels, and are recognised to deter insect herbivory (Boyd, 2009; Mogren and Trumble, 2010; Stolpe and Muller, 2016). Works in lepidoteran pests reported mixed responses depending on insect species, but feeding deterrence tends to increase with increasing metal concentrations such as 50–200 mM for $CuSO_4$ (El-Bassiouny, 1991) or > 0.1 % (~ 1,000 ppm) for $ZnSO_4$ (Sell, 1971; Pollard and Baker, 1997). Concentrations of metals in natural bee food are unlikely to be as high as 200 mM Cu (~13,000 ppm), but compared to other metals, bees were most deterred by the presence of $CuCl_2$ in solution in all the conditions tested (Figure 3.5). Hladun et al. (Hladun *et al.*, 2012) tested the gustatory responses of honey bees to Se in 1.0 M sucrose solutions delivered as either selenate or selenomethionine. These authors found that Se was aversive dependeding on the form of Se provided; bees exhibited fewer PER responses to higher concentrations of selenomethionine, but not selenate. In drinking

assays, bees did not detect Se specifically; bees consumed food with as much as 6,000 ppm selenate and the number of bees consuming these solutions did not differ from sucrose control (Hladun et al., 2012). In contrast to (Hladun et al., 2012), I found that forager bees detect other metal nutrients in nectar-like solutions through antennal taste (Figure 3.5 a), but are less likely to consume these solutions when high in either Fe^{2+} or Cu^{2+} (100 mM, > 5,000 ppm). This scenario is similar to rejection of nectars high in K and P (Waller, Carpenter and Ziehl, 1972; Afik, Dag and Shafir, 2008; Afik et al., 2014). Gustatory responses to salts/metals were not uniform, i.e. high responses to low concentrations and low responses to high concentrations, but were rather dependent on mineral type. Furthermore, anion properties are known to influence gustatory responses to salt in both insects and rats (Gillary, 1966; Breza and Contreras, 2012). This may explain in part the difference between bee responses when stimulated with organic or inorganic Fe salts (see Figure 3.6), indicating that organic anion (e.g. citrate) was more phagostimulatory than inorganic anion (e.g. chloride).

3.6.3 Bees showed limited gustatory responses to increasing concentrations of Na in solution

The third and last aim was to test whether 1.0 M sucrose (30 % w/v sucrose) could mask the taste of mineral salts at low appetitive levels (Figures 3.4 b, d and 3.5 b, d). High concentrations of Na were expected to reduce gustatory responses and rejection (Hagler, 1990; De Brito Sanchez *et al.*, 2014). I then tested the effect of a sucrose gradient on the gustatory perception of increasing concentrations of Na. As expected, taste sensilla on the antennae were highly responsive to sucrose solutions (Haupt, 2004; Scheiner, Page and Erber, 2004), compared to the proboscis. Bees responded highly to all sucrose solutions regardless of Na concentration (Figure 3.8 a). GRNs housed in antennal sensilla produce more intense responses at lower sucrose concentrations (<0.1% or 2.9 mM) (Haupt, 2004; Simcock, 2014; Jung et al., 2015) compared to both the mouthparts (Whitehead and Larsen, 1976a; Whitehead,

1978) and the tarsi (De Brito Sanchez *et al.*, 2008, 2014). Here, I did not see an increase in PER proportional to increased sucrose concentrations as previously predicted; nor I find a deterent effect of high Na (e.g 1.0 M; 2.3%; 22,989 ppm Na^+) when presented in lower sucrose rewards (e.g 0.1 M sucrose solutions) to the antennae. Pollen and water foraging honey bees detect low sucrose concentrations (0.1%; 0.003 M) and, thus, exhibit high sucrose PER responsiveness, compared to nectar foragers (Page, Erber and Fondrk, 1998; Scheiner, Page and Erber, 2004).

Surprisingly, the presence of Na in solution did not increase its phagostimulatory power, except compared to water alone (Figure 3.4 and 3.8). Moreover, elevated concentrations of Na as high as 1.0 M also did not trigger rejection in both antennal PER and drinking assays (Figure 3.8). Repeated antennal stimulation may have induced behavioural sensitization to increasing concentrations of Na (Figure 3.8 a). However, this ceiling effect was similarly observed for all solutions varying in sucrose concentration. These assays were conducted in late summer (September) and early fall (October), therefore is possible that the internal state of these forager bees influenced the gustatory responses to sodium. Bonoan et al. reported that mineral preferences in free-flying forager bees change with the season (Bonoan *et al.*, 2016).

Unexpectedly, increasing levels of Na in sucrose solutions did not induce an increase in the likelihood of consuming the droplet of solution (Figure 3.8 b) nor a decrease at high concentrations (Figure 3.8 b). The acceptance thresholds for sucrose solutions was above 0.1 M sucrose (4.0 % w/v). However, there was no clear acceptance – rejection threshold for Na within the broad range of concentrations tested (0–22,989 ppm Na^+) regardless of background sucrose concentration (Figure 3.8 b). In previous studies, water foragers demonstrated high antennal PER to 1.5% NaCl in water solutions (Lau and Nieh, 2016), which is above the average concentrations found in natural water sources. Avocado nectars were reported to contain low Na (54 ppm), but high K (> 3,000 ppm) that was the likely cause of nectar rejection by bees (Afik, Dag and Shafir, 2006). Here, it was not clear whether the decrease in consumption was due to the low sucrose or Na concentration. At the

level of the proboscis, bees are more sensitive to changes in food mixtures and associated gustatory cues. Also, as shown in Figure 3.4 d, bees were not deterred by high Na in sucrose solutions. Altogether, these results are not conclusive in realtion to the masking effect of sucrose on the oresence of Na in solution. A masking effect of sucrose would be accepted, for example, if high levels of Na would be accepted in increasing concentrations of sucrose, but otherwise rejected in water or lower levels of sucrose. In the drinking assays, water and low sucrose (0.1 M) solutions (regardless of Na concentration) were not phagostimulatory enough to be drank (Figure 3.8 b). Another approach to assess the potential masking effect of sugars on the gustatory perception of minerals could be testing salt in ssolutions of two different sugars in a broader gradient of concentrations and during the summer season. A discrimination assay (e.g. differential gustatory conditioning) using two salts in a gradient of sucrose solutions could provide more substantial information regarding the behavioural gustatory inputs of minerals in nectar-like solutions.

3.7 Conclusion

Taken together these results along with previous reports confirm that forager honey bees are attracted to low levels of major salts in water, but deterred by high concentrations in nectar-like solutions. I found that forager bees (mixed ages and, possibly, foraging tasks) are able to detect salts and metals in solution with responses varying according to mineral identity and dietary context (water/sucrose solution). This suggests that different salts and metals may evoke different taste sensations, possibly associated with distinct biological significance. It is worth noting that the range of concentrations used here in the first and second experiments is still high and may have induced a certain degree of behavioural sensitization. To the best of my knowledge this is the first study addressing behavioural gustatory inputs and ingestion of metal nutrients. Metal detection seems to be species and concentration dependent in greater extent compared to salts. This is not surprising as exposure to high concentrations of metals can affect biological processes such as growth,

development and fertility in insects, which may be associated with cumulative oxidative damage induced by reactive oxygen species (Betteridge, 2000). Furthermore, the antennae appear to detect metals at low phagostimulatory levels. In higher concentrations, these metals are promptly flagged by taste sensilla on the mouthparts and solutions are rejected.

By performing these gustatory assays across a broad range of essential minerals, I found evidence that bees detect and respond positivelly to low concentrations (< 200 ppm) of both salts and metals in water solutions with antennal PER giving the best results compared to water control. Also, I found no specific rejection of concentrated saline solutions either upon an antennal stimulation or mouthparts. Yet, bee mouthparts appear to be more sensitive to salts/metals detection either in water or sucrose solutions with high Fe and Cu as mineral stimuli induced the greatest decrease in drinking responses. Though I did not find a strong evidence for the masking effect of sucrose on the detection of Na in solution, results indicate that 1.0 M sucrose may influence taste of low salt/metals because and across all treatments, only high Fe and Cu in 1.0 M sucrose solutions were sufficiently distastefull to be rejected by bees. Using a mixture of mineral salts would be a more realistic approach, however, I used a minimalist study, with single salt solutions to assess a specific behavioural response concerning the presence/absence of a particular element. Further and more detailed studies are necessary to disentangle what remains inconclusive.

The present study sheds some light on the behaviour of mineral feeding in relation to pre-ingestive regulation. Behavioural regulation of mineral salt intake will be addressed in the following Chapter 4, which covers the next approach to understand the feeding responses to dietary minerals by evaluating consumption and preference-aversion thresholds in free-flying bee cohorts.

3.8 Acknowledgments

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4

Self-Preference and Behavioural Regulation of Mineral Salt Intake in Young Worker Bees

Chapter 4

Self-Preference and Behavioural Regulation of Mineral Salt Intake in Young Bees

4.1 Highlights

- Young bees show distinct preferences for mineral solutions containing important dietary salts/metals.
- Young bees perceive and avoid ingesting solutions with high levels of salts and metals to prevent intoxication.
- Consumption of Fe and Cu diets were regulated according to Bertrand's rule
- High Cu (500 ppm) induced the highest mortality recorded (40%) in these experiments.
- In contrast to all the other mineral treatments, bees preferred high Na diets.

4.2 Abstract

Optimal nutrition is only attained through the regulation of ingested nutrients. Nurse honey bees ingest and process food to feed all other members of the colony including the single reproducing female queen and the developing larvae. Nectar carbohydrates, pollen proteins and fats supply most of bees' nutritional requirements. Mineral salt intake occurs mostly by pollen feeding. Other insects have been observed to optimise salt intake by regulating ingestion between low and high salt mixture contents. An approach which tests whether honey bees regulate their intake of salts and metals has not been reported yet. In the previous Chapter 3, data showed that gustatory responses of forager bees were dependent on the mineral type. Moreover, the extent of gustatory perception was associated with the dietary context of minerals (e.g. background solution). Overall, these results showed that forager bees (stimulated once) were able to detect certain minerals in solution by producing appropriate behaviours that informed whether a solution was appettive or aversive. In the current Chapter, I aim to assess the feeding responses to dietary minerals by evaluating consumption and preference-aversion thresholds over time in free-flying young bee cohorts. The concentration of single minerals in 1.0 M sucrose solutions were, therefore, manipulated to ascertain acceptance-rejection feeding thresholds for K, Na, Ca, Mg, Fe, Zn, Cu and Mn. By means of two-choice feeding assays, I assessed whether bees preferred certain levels of mineral-enriched over mineral-free solutions. I also measured the effects of mineral diets on bee survival. Using this framework, I predicted that palatable diets are consumed and preferred, but distasteful are avoided promplty. Moreover, by adjusting consumption between the two diets, bees can regulate mineral intake . I found that young bees preferred Na, Fe, Cu diets compared to sucrose alone, thus demonstrating different feeding preferences across mineral salts. Na and Fe-diets significantly affected total diet ingested by young bees compared to sucrose only diet. Bees rejected high

concentrations of all the minerals tested, except Na solutions. Young bees fed with mineral diets survived well even at very high levels of concentration. These data, therefore, indicate that adult honey bees regulate the intake of mineral salt diets and may display homeostatic mechanisms for regulating mineral intakes and attain better nutrition.

This study is the first to show that bees optimise their intake of micronutrients and adjust their behaviour to avoid intoxication when salts/metals are in excess (Bertrand's rule).

4.3 Background

Food ingestion is a vital behaviour observed in all animals and involves the acquisition of chemical elements from diet, which supply the nutritional demands imposed by biological processes. Nutrition in social insects, and honey bees in particular, is critical as different feeding regimes (quality and quantity) control not only caste development and reproductive differentiation (queen or worker) (Kucharski *et al.*, 2008), but also caste differentiation and behavioural plasticity between adult workers (nurses to foragers) (Crailsheim *et al.*, 1992; Schulz, Huang and Robinson, 1998; Amdam *et al.*, 2004; Toth and Robinson, 2005).

Nectar and pollen collected from flowers by forager bees are the main food sources for honey bees that satisfy their nutrient requirements (Haydak, 1970; Brodschneider and Crailsheim, 2010). Nectar is a rich source of carbohydrates, whereas pollen is virtually the exclusive source of non-carbohydrate nutrients for bees (proteins, amino acids, lipids, vitamins and mineral salts) (Haydak, 1970; Brodschneider and Crailsheim, 2010; Nicolson, 2011). Akin to other insects, developing larvae and the reproducing females have high demands for protein and pollen nutrients, but adult bees rely mostly on a sugar-based diet to maintain their own energetic demands and temperature homeostasis within the colony, and to support flight. Young adult workers also consume pollen for somatic maintenance,

but mostly to support hypopharyngeal glands (HPG) development, which produce glandular secretions like royal jelly to feed their larvae and the queen (Crailsheim *et al.*, 1992). Young nurse bees (0-12 days old) are, thus, pivotal in mediating colony nutrition by both producing nutrient-rich jelly (Crailsheim *et al.*, 1992; Hrassnigg and Crailsheim, 1998) and distributing the digested nutrients among nestmates (Brodschneider *et al.*, 2017) via trophallaxis (Crailsheim, 1998).

Pollen composition (quality and quantity) influences adult bee health, physiology, immune function and lifespan (Alaux *et al.*, 2010, 2017; Di Pasquale *et al.*, 2013; Frias, Barbosa and Lourenço, 2016), as well as colony survival (Smart *et al.*, 2016). Whereas the nutritional value of pollen often relates to its protein and amino acid contents (De Groot, 1953; Cook *et al.*, 2003; Nicolson and Human, 2013), pollen is also the major source for mineral salts, including K (potassium), P (phosphorous), S (sulfur), Mg (magnesium), Ca (calcium), Na (sodium), Fe (iron), Zn (zinc), Cu (copper), Mn (manganese), and other trace elements such as Se (selenium) and Al (aluminium) (Somerville and Nicol, 2002; Morgano *et al.*, 2012; Filipiak *et al.*, 2017); see also (Black, 2006).

Minerals are important for osmoregulation (Nicolson and Worswick, 1990) and to sustain metabolic and tissue functions as they act as cofactors of metalloenzymes (~1/3 of all enzymes known so far) (Hoppert, 2011) and metal-responsive transcription factors (Günther, Lindert and Schaffner, 2012); for a review in insects see also (Dow, 2017). For instane, insects require K, Mg, and P during development (Dadd, 1973; Perkins *et al.*, 2004), and Na to increase the reproductive output of adults (Smedley and Eisner, 1996; Walker, Corrales-Carvajal and Ribeiro, 2015). In honey bees, it is generally assumed that mineral levels in pollen are sufficient to support development and growth (Haydak, 1970; Brodschneider and Crailsheim, 2010). For example, some have inferred mineral requiremnets of bees by quantifying the mineral composition of either bee-collected pollen or bee bodies (Manning, 2002; Somerville and Nicol, 2002; Black, 2006; Manning, 2016). However, mineral pollen contents can be highly variable, 1–7% in minerals (Lunden, 1954) or

2–4 % in ash (dry matter) (Nation and Robinson, 1971b; Herbert and Shimanuki, 1978).

The specific dietary requirements for mineral nutrients of adult honey bee workers are not well understood and have been rarely studied. The few studies that exist are from 40–50 years ago; one suggested that minerals are not essential for the development of hypopharyngeal glands in young nurse bees (Haydak and Dietz, 1965). Yet, two important studies to date showed that bees need a specific range of micronutrients in diet; these researchers reported that a systematic increase of pollen ash in synthetic diets reached the best results for brood rearing and worker lifespan at 0.5–1% pollen ash (Nation and Robinson, 1968; Herbert and Shimanuki, 1978). A more recent meta-study reported a mismatch between mineral body contents for different castes (queens, drones and workers) and bee-collected pollen composition, especially for Na (Filipiak *et al.*, 2017). Based on these data, these authors suggested that honey bee diet is deficient in specific mineral nutrients. Others reported that elevated concentrations of minerals may induce dysentery (Imdorf *et al.*, 1985; Crailsheim and Pabst, 1988) and muscle paralysis (Horn, 1985). Nutrient imbalances are, therefore, common because most animals find it difficult to consume foods that are chemically balanced and that contain the adequate proportions of all nutrients (House, 1969); see also (Simpson and Raubenheimer, 2012). It is, thus, not surprising that "the right dose differentiates a poison from a remedy" (Paracelsus, 1965). This duality has been formalised into a dose-response model to express the relationship between deficiency-toxicity of micronutrients, termed– Bertrand's rule (see Figure 1.4, Chapter 1). This model assumes that an increase in health benefits occurs with the ingestion of low levels of a nutrient until an optimum threshold; further ingestion translates in increased costs as the regulatory mechanisms become overwhelmed and excesses become toxic and potentially lethal (Bertrand, 1912; Raubenheimer, Lee and Simpson, 2005). This relationship reflects the fact that nutrient intake is optimised around values that promote fitness and survival (Trumper and Simpson, 1993; Simpson *et al.*, 2004); see also (Simpson and Raubenheimer, 2012). Optimisation of nutrient intake for adequate nutrition can, therefore, be attained through

mechanisms such as preingestive (taste) and postingestive metabolic sensors that function together to regulate nutrient intake; for review see (Behmer, 2009; Simpson and Raubenheimer, 2012). Bees must forage and perceive the quality of food in its various dimensions to adjust nutrient supply to demands. While interest in mineral detection and bee nutrition is growing (Lau and Nieh, 2016; Bonoan *et al.*, 2017), how and whether honey bees regulate the ingestion of minerals has not been formally studied. Adult bees can adjust and regulate their macronutrient intake to buffer optimal fitness traits against high nutrient variation (Altaye *et al.*, 2010; Pirk *et al.*, 2010; Paoli *et al.*, 2014; Stabler *et al.*, 2015; Vaudo *et al.*, 2016, 2017). Under laboratory settings, honey bees were also reported to adjust nutritional imbalances by selecting diets that compensated experienced deficiencies (Hendriksma and Shafir, 2016). These studies indicate that both at the individual and at the colony level, honey bees can adjust their feeding behaviour to control food and specific nutrient intake.

Similar behavioural mechanisms may as well be employed by adult workers to ingest limiting minerals, while avoiding excesses that induce dehydration and may lead to death. Seminal work in locust nymphs demonstrated that behvioural regulation of salt intake occurs though adjusting consumption between low and high salt mixture contents (Trumper and Simpson, 1993, 1994) and rejection of toxic high salt diets (Bernays and Lee, 1988). Therefore, understanding whether worker bees detect specific minerals in food and regulate its ingestion warrants attention. To my knowledge, no study has yet addressed the role of mineral salts in adult bee nutrition, specifically whether young workers, as mediators of larvae and adult nutrition, regulate the ingestion of mineral salts within ranges present in pollen. This study aims to identify a suitable range of concentrations that supports feeding and survival for the eight most prevalent minerals in pollen (Na, K, Mg, Ca, Fe, Zn, Cu, Mn) using two-choice assays in laboratory settings. Despite all these nutrients are vital for proper metabolic function, it is possible that requirements for each mineral differ in magnitude and, therefore, inducing different feeding responses in honey bees.

I designed this study addressing the individual effects of minerals on the feeding responses of young workers. Using the methods described in earlier chapters, I was able to verify whether young workers demonstrate regulation of mineral salt ingestion through behavioural adjustments in consumption. This work is the first to evaluate dietary self-selection of metal nutrients, and to assess salt preferences of young adult workers in a choice context. In this study, I employed the Bertrand´s rule concept (Figure 1.4, Chapter 1) to predict preference-rejection thresholds and behavioural regulation of mineral intake. This rule stems from the dose-response curve for essential mineral nutrients. At low doses, increased intake associates with health benefits until an optimal intake is reached; further intake at higher doses results in health costs (Bertrand 1912, Mertz 1981).

4.4 Methods

4.4.1 Experimental Animals

Honey bee colonies from Buckfast strains were kept at the Newcastle University campus between March to October 2014-2016. Young (nurse) bees process and feed developing and reproducing bees (Crailsheim *et al.*, 1992; Lass and Crailsheim, 1996; Toth and Robinson, 2005; Wang *et al.*, 2014), thus, are expected to regulate food in a way that meets their own needs and the requirements for the production of royal jelly. Suitable brood frames with capped cellls were, thus, marked and selected from colonies in the apiary. Within two days before estimated eclosion, marked brood frames were shifted indoors and transferred to a ventilated incubator (Sanyo MIR-553) set at 34º C in the dark to mimic natural field conditions inside the (Winston, 1991). Brood frames were checked every day for emerging bees. Newly-emerged bees (0 up to 30 h old) were brushed off the frames to a large ventilated container. Bees were then randomly counted and assigned to experimental

boxes to make-up bee cohorts, later allocated to feeding treatments (Figure 4.1). At the end of the experiment, bees remaining alive were frozen-killed.

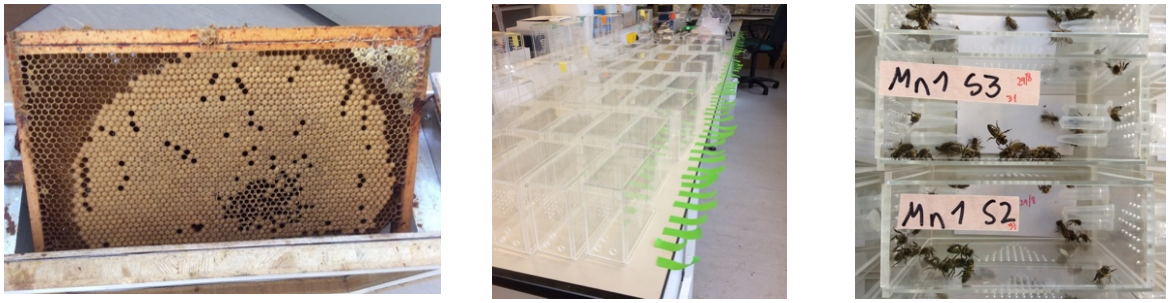

Figure 4.1. Experimental animals for feeding cohorts. Previously selected combs containing sealed brood were used to collect bees upon eclosion. Then, newly emerged bees were randomly assigned to experimental boxes used in feeding assays. Each box is a unit replicate for each feeding treatment and included N=30 bees

4.4.2 Chemically-defined Diets

Worker bee survival in lab conditions can be ensured by providing sufficient sucrose solutions at 30–50% w/v and, thus, used as standard food in the laboratory (Barker and Lehner, 1978; Brodschneider and Crailsheim, 2010; Williams *et al.*, 2013). This sucrose concentration in the range of nectar sugar concentrations that bees encounter naturally in floral nectars (Nicolson and Thornburg, 2007; Brodschneider and Crailsheim, 2010). Therefore, chemically–defined liquid sucrose diets were taken as the base control diet to sustain adult honey bees over the course of the experiments in feeding assays. Therefore, for brevity, hereafter 'mineral salts 'or only 'minerals' refer to all micronutrients tested (both salts and metals). Salts only refer to macroelements (sodium (Na), potassium (K), calcium (Ca), magnesium (Mg)) and metals denote microelements (iron (Fe), zinc (Zn), copper (Cu), manganese (Mn)), respectively. All minerals derived from inorganic compounds, specifically chloride conjugates. Diet concentrations were tailored for each mineral type. The range of concentrations was drawn after (Herbert and Shimanuki, 1978) and based on values present in bee-collected pollen (refer to Table 1.4, Chapter 1 for mean bee

pollen concentrations). Exploratory range-finding studies confirmed these concentrations as described in Chapter 2, Study 1.

Diet solutions were primarily formulated using reagent grade sucrose at 1.0 M (34.2 % w/w) dissolved in distilled water (pH≈ 6.5). Sucrose solution alone was taken as the control feeding treatment (no minerals added) (for reagent details see S1 Table, Appendix A). To make up mineral diets, the analyte cation (positively charged ion) was used to calculate the concentration (e.g. Molecular Weight (NaCl) * [(Na^+ $mgmL^{-1}$/ atomic weight (Na^+)]. Parts per million (ppm) was the unit concentration used in mineral diet treatments in the current Chapter. Solutions were very dilute and as such, for simplification of unit conversions I considered: 1ppm = $1mgL^{-1}$ (solution density = water density = 1 gmL^{-1}); see Table 4.1.

A stock solution for each mineral type was prepared and subsequently used to make up other mineral treatments by serial dilution. All stocks and working solutions were freshly prepared and kept at -20º C prior use. Diet solutions were defrosted, homogenized and provided fresh every day over the course of the experiments.

4.4.3 Feeding Boxes and Feeding Tubes

Feeding units comprised cohorts of ~30 newly emerged bees housed in customized acrylic ventilated boxes (dimensions: 13 x 11 x 4 cm; 0.4 L capacity) with slide front and back doors (Bay Plastics, Ltd., UK). Boxes were randomly assigned to different treatments, and each was taken as one unit replicate per treatment (Figure 4.2, Bee Feeding unit). Each lateral side of the box displayed three holes (Ø 10.9 mm) to insert modified 2.0 mL-microcentrifuge Eppendorf tubes (211-2120, VWR International) used as feeding tubes. Each tube was modified by drilling 3-4 holes (Ø 2.0 mm) in line and 5 mm apart. A piece of paper was placed at the bottom of each box to visually account for defecation extent between feeding treatments and over the course of the experiment. No formal measurement was performed to account defecation rates.

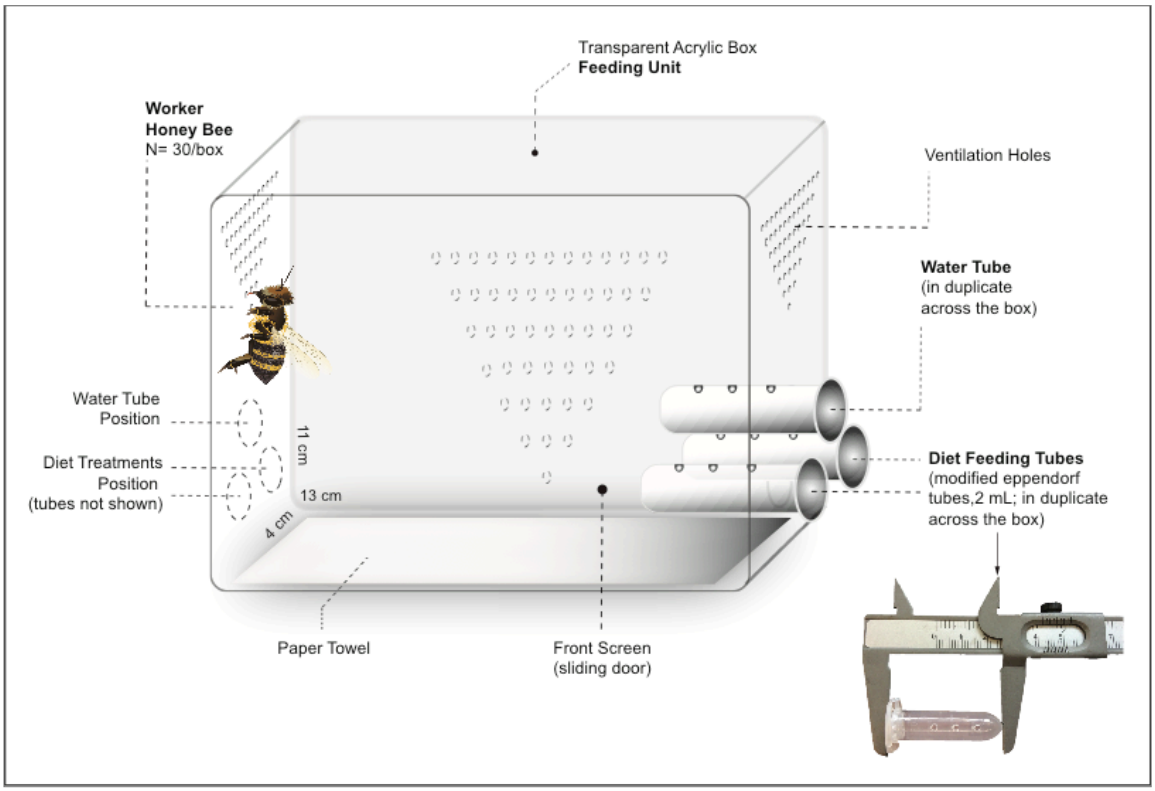

Figure 4.2 Experimental Box Layout – Bee Feeding Unit – used in two-choice feeding assays. Each unit was composed of three pairs of feeding tubes, one paper towel at the bottom and N=30 honey bees. For simplification only 3 tubes are displayed on one side of the box. Each side of the box delivers water (one tube) and a single diet (two tubes) either control or treatment diet. Graphics are not at scale. @ Credits to Almudena Clemente for bee artwork.

Feeding tubes were replaced daily with fresh diet. This hampers diet contamination by environmental dust, which could induce a build-up in trace elements or microbial growth (Williams *et al.*, 2013). Feeding tubes were provided in duplicate per diet per box (Figure 4.3). This ensured that bees did not run out of food between measurements.

Studies using laboratory feeding assays often disregard mentioning how feeders are positioned and manipulated over the course of the experiments. Therefore, in preliminary experiments described in Chapter 2 (Study 4), I assessed whether tube position within the feeding box affected the magnitude consumption and food choices, and also the effect of moving the tubes around every day, i.e. by shifting diet tubes laterally every day in the side-by-side set-up. I tested two delivery regimes (side-by-side vs. crosswise regime, Figure 2.10 a) and found that tube position and location had a significant impact on the magnitude of measured

consumption, but not on feeding preferences (Figure 2.10 b and c); for further details see Chapter 2, Study 4. Though, the side-by-side tube regime was more labour-intensive and possibly more prone to human error, it yielded higher magnitude consumption measurements (Figure 2.10 b). This increases the reliability of consumption measurements in treatments that induce high bee mortality (e.g. see Chapter 2, Study 1, Figure 2.5 g). The number of bees in each feeding box affects the measurement of food consumption (see Chapter 2, Study 5, Figure 2.11). For food delivery, therefore, I decided to use the side-by-side regime with treatment position daily switch (Figure 4.3).

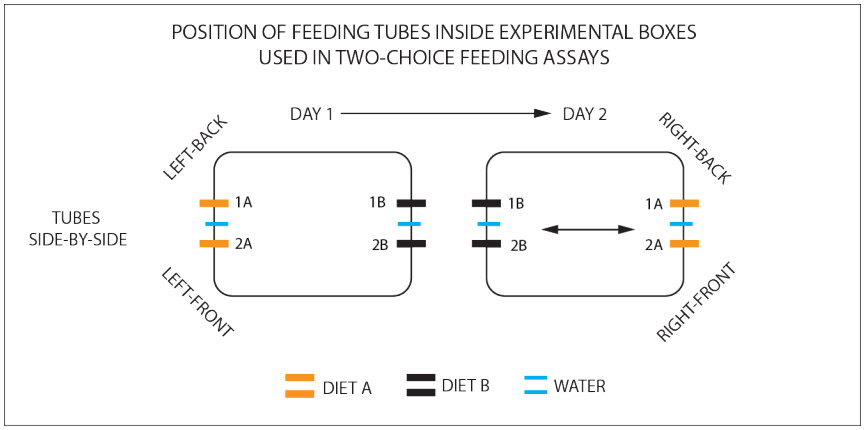

Figure 4.3 Position of feeding tubes – side-by-side tube layout. Diet tube pairs switched sides across the box every day, i.e. on day 1, a single diet tube pair (1A and 2A) was displayed on the left-side of the box, whilst the other pair (1B and 2B) was displayed on the right, and *vice-versa* on day 2. This method also intended to measure the effect of moving the tubes around within feeding boxes.

Every day, each pair of diet tubes was placed at the opposite side of the box to prevent bees from spatial bias when consuming from each treatment. For example, diet A was delivered on the left-hand side of the feeding box on day 1, but on day 2, freshly replaced diet A was offered on the right-hand side of the box. A pair of distilled water tubes (one at each side of the box, see Figures 4.2 and 4.3) was always provided and measured. In total, six feeding tubes were provided per experimental box (Figure 4.2). A water source should be always provided in feeding experiments. Water is a source of minerals (Brodschneider and Crailsheim, 2010) for honey bees, and thus, distilled water was preferred over tap/mineralized water, which could

input confounding variables. In the sucrose control treatment boxes (no mineral nutrient added), four tubes were used instead of two. All experimental variables were conserved among treatment groups except for the variable of interest.

4.4.4 Feeding Treatments and Conditions

Choice assays were used in this study to investigate: 1) self-selection and non-randomness of food intake; 2) how an animal regulates the ingestion of individual minerals varying in concentration; 3) the effects of mineral type and concentration on adult bee survival in lab conditions when able to self-select food. Here, bees were given a choice between two diets: sucrose only diet (mineral-free) paired with mineral-laced sucrose diet.

After assigning bee subjects to empty experimental boxes, feeding treatments were commenced by adding replenished feeding tubes with respective diets. Newly emerged bees were starved for 2–5 h. The initiation of feeding treatments was randomised every time using the sample() function in RStudio Software (RStudio Team (2016). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA; URL: http://www.rstudio.com/). This prevents random errors (e.g. risk of mortality by starvation in later initiated feeding treatments) occuring in the same treatments.

Forty feeding treatments were assayed and derived from eight minerals of inorganic nature (chlorides) (salts: Na, K, Ca, Mg and metals: Fe, Cu, Zn, Mn) tested at five levels of concentration: 0 (control), + (low), ++ (medium), +++ (high), ++++ (very high) (Table 4.1). Each feeding treatment respected one concentration only of a single mineral nutrient (e.g. Na, sodium). Feeding treatments were conducted and measured independently up to 7 days after bee collection.

Feeding treatments comprised a minimum of N= 4 and a maximum of N= 10 boxes (150-300 bees/treatment) that offered a choice between sucrose only diet (control diet) and one single salt/metal-enriched sucrose solution. Diet solutions were replaced by fresh diet every day. Water was also provided and replenished by fresh water daily.

Sucrose only treatments, in which bee cohorts were fed sucrose only solutions, are defined as the control feeding treatments (no salt/metal). Control treatments were conducted for each group of treatments as they can indicate the nutritional and physiological state of adult honey bees and be compared across mineral feeding treatments. Behaviour and physiology of bees can vary across the season and caste (Williams *et al.*, 2013).

Mock evaporation boxes (same set-up; bee-free) were used in the experimental designs to account for the evaporation loss of treatment solutions. For each feeding treatment, conditions for mock boxes were kept the same (e.g. box and tube type and set-up, type of diet, lab conditions and frequency of measurements). Each feeding treatment was attended by a minimum of N= 2 and a maximum of N= 4 mock boxes. Experimental and respective mock boxes were kept in the incubator chamber at 34º C in the dark during the experiments (Chapter 2, Figure 2.3) and shuffled inside the incubator every day to minimize bias on evaporation rates of diets (for details refer to Chapter 2, Study 3). Temperature and relative humidity probes (OM-EL-USB-2) were maintained inside the incubator chambers and programmed to monitor experimental conditions throughout the season and while performing experiments (S3 Figure, Appendix A). For further equipment details refer to S8 Table, Appendix A.

Hereafter, a colour code is attributed to each salt/metal to ease contextualization of the graphics when necessary. As such, **sucrose control** diets are shown in blank, **sodium (Na)** diets are depicted in grey, **potassium (K)** in yellow, **calcium (Ca)** in brown, **magnesium (Mg)** in red, **iron (Fe)** in orange, **copper (Cu)** in aqua blue, **zinc (Zn)** in green and **manganese (Mn)** in magenta hues.

From feeding assays, two main variables were measured daily over the course of the experiment 1) diet consumption and 2) number of dead bees.

Table 4.1 Mineral-enriched sucrose diets in feeding assays. Control diet refers to 1.0 M sucrose solution alone. Each mineral is depicted by a colour code.

Cation	Diet Levels	Diet treatments	[ppm]	[mM]
Sodium (Na^+)				
	+	Na5	5	0.22
	++	Na50	50	2.18
	+++	Na100	500	21.7
	++++	Na1000	1,000	43.5
Potassium (K^+)*				
	+	K10	10	0.26
	++	K100	100	2.56
	+++	K1000	1,000	25.6
	++++	K10000	10,000	256.
Calcium (Ca^{2+})				
	+	Ca1	1	2.50×10^{-2}
	++	Ca10	10	0.25
	+++	Ca50	50	1.25
	++++	Ca500	500	12.5
Magnesium (Mg^{2+})				
	+	Mg10	10	0.41
	++	Mg30	30	1.23
	+++	Mg300	300	12.3
	++++	Mg3000	3,000	123.
Iron (Fe^{3+})*				
	+	Fe1	1	1.79×10^{-2}
	++	Fe10	10	0.18
	+++	Fe100	100	1.79
	++++	Fe1000	1,000	17.9
Zinc (Zn^{2+})*				
	+	Zn0.5	0.5	7.65×10^{-3}
	++	Zn5	5	7.65×10^{-2}
	+++	Zn50	50	0.77
	++++	Zn500	500	7.65
Copper (Cu^{2+})*				
	+	Cu0.5	0.5	7.87×10^{-3}
	++	Cu5	5	7.87×10^{-2}
	+++	Cu50	50	0.79
	++++	Cu500	500	7.87
Manganese (Mn^{2+})				
	+	Mn1	1	1.82×10^{-2}
	++	Mn10	10	0.18
	+++	Mn50	50	0.91
	++++	Mn500	500	9.10

*K, Fe, Cu and Zn concentrations are in logarithmic scale base 10.

4.4.5 *Assessing self-preference, daily and total consumption of young worker bees fed mineral diets*

To assess whether young bees prefer diets containing minerals over mineral-free diets (sucrose control), and to identify a range of acceptance-rejection feeding thresholds for each salt/metal, I conducted two-choice feeding assays over bees' first 1–7 days.

Each pair of diet tubes was pooled and weighed together, assuming there is no major differences between tubes. For each treatment, diet consumption was measured daily by weighing mass reduction from feeding tubes of each diet/box. Differences in mass weight (g) per diet recorded every 18–28 h were considered the daily raw consumption per box. Mock evaporation boxes, were measured each day to account for mass reduction by evaporation loss. Diet tubes respecting the same feeding treatment and diet were also weighed together and later divided by the number of mock box replicates. The average % of mass reduction by evaporation over the course of the experiment was calculated per diet per box to correct raw consumption for each bee unit. Water tubes were processed similarly. For details refer to Chapter 2 – Study 3, and see S10 and S11 Figures, Appendix A). Please refer to Chapter 2, Study 3 for a description of evaporation correction methods used. Daily raw consumption was then corrected for evaporation losses and converted to volume units using solutions' density (S12 Figure, Appendix A). Daily volume consumption was finally divided by the number of live bees in each box and averaged over 6 days.

To better interpret the patterns of feeding, the preference index (PI) was calculated from the final volume consumption figures for each treatment. For each diet treatment and concentration, the equation used was as follow: PI= [(mineral diet consumed) – (sucrose diet consumed)]/ total diet consumed. Total diet refers to the sum of volumes consumed form both treatment and control diets (excluding water). Preference indexes were calculated either as total PI (over 6 days) or daily PI for each treatment. To assess how diet consumption fluctuates over 6 days, daily diet

preferences were estimated as PI/day. Water consumption was measured and corrected for evaporation loss, but analysed separately.

Consumption measurements were recorded using an analytical balance (Sartorius QUINTIX 64-1S, datalogger) connected to a laptop. Raw consumption values were directly recorded into MS Excel worksheets (Microsoft Office Software, 2015). Time of measurements was recorded daily for each feeding (see S9 Figure, Appendix A).

4.4.6 Testing the effects of mineral diets on the survival and fresh body weight of young worker bees

To assess the impacts of mineral feeding on the survival of young bees across the full range of mineral salts tested, the number of dead bees in each experimental box was recorded daily and removed. Honey bee mortality was used as a proxy for health costs associated with active nutrient ingestion. Honey bees found dead were removed to avoid spread of pathogens. The sucrose only treatment was used as the reference treatment to compare survival curves between feeding groups for each salt/metal.

As a secondary health parameter, the fresh body weight of five bees per box per treatment was measured (N= 50 bees/treatment). Because of the difficulties attached to periodic recording of weights, I chose to record weight only at the end of the experiment (day 6). For comparison, untreated worker bees (no feeding treatment received) directly collected from brood frames were also weighted (FW, fresh body weight).

4.4.7 Data Processing and Evaporation Loss Adjustments

Raw consumption values (g) obtained from daily weight reduction (e.g. Δday1 = day1pre-weight - day1post-weight) per diet per box followed a series of calculations until the final corrected consumption was reached. Because diet

treatments consisted of liquid diets prone to evaporation losses, each raw consumption value (g) was corrected for evaporation loss using volume losses recorded from respective mock boxes. In preliminary experiments described in Chapter 2, a series of methodologies were employed to assess the effect of different adjustments on the magnitude of mean consumption figures. I found that the mean evaporation loss (%) correction (Method E, Chapter 2, Study 3) provided a better correction methodology for experiments of this kind. This adjustment did not affect negatively the magnitude of mean volume consumption (Figure 2.7, Chapter 2). In contrast to other traditionally-used methods that involve direct subtractions and a certain degree of data manipulation, the mean % loss of solution by evaporation is a normalised value that adjusts the whole data set. The mean % loss of solution by evaporation over the course of the experiment was calculated as [(Δevap)/#mock boxes)*100/(evap.pre-weight/box)] (evaporation correction method E; for further details refer to Chapter 2, Study 3). The mean % loss per diet per box was then subtracted to the raw consumption value per diet per box (see S10 and S11 Figures, Appendix A).

After adjusting for evaporation loss, mass (g) values were converted to volume by multiplying by solution's density using the relationship [volume (mL) = mass (g)/ density (gmL-1)]. Densities (and pH) of experimental solutions were measured and the average density figure used to convert mass to volume (see S12 Figure, Appendix A). This volume of consumed diet (mL) was then divided by the number of live bees per box per day. Volume (μL) per bee per diet per box was the final consumption figure used for statistics and plotting. Based on preliminary experiments, which tested the reliability of consumption measurements in relation to experimental conditions (e.g. number of bees/box and evaporation loss), two cut-off thresholds were proposed and further used to process data (refer to Chapter 2, Study 5 for further details). As such, a minimum number of bees in one box was defined to be at least 20% of the total number of bees, but no less than 6 bees/box at a certain day (e.g. N= 30 bees/box from which 20% is N= 6 bees/box) (see Figure 2.11, Chapter 2). Second, a minimum number of reliable box replicates in a certain day (N= > 6

bees/box) was established as 30% of the total number of box replicates (e.g. N= 10 boxes/treatment from which 30% is N= 3 boxes/day). I reasoned that by employing these two rules when processing consumption data provides more reliable measurements and increased confidence in results.

4.4.8 Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics for Macintosh (version 24.0, 2017) and graphs depicted using GraphPad Software, Inc. (Prism 5 for Mac OS X, version 5.0a, 2007). Total preference indexes and volume consumption per treatment (μL/bee) were analysed using Generalized Linear Models (GzLM) fitted for the appropriate data distribution. A factorial model was first constructed to test the effects of mineral type and concentration on total diet preferences/consumption/bee.

Salts and metals were analysed separately. Then, one-way GLzM models were applied to each of the mineral treatments in the choice feeding assay for pairwise comparisons across concentrations. Post-hoc comparisons were performed using Sequential Bonferroni. This method is less conservative and more powerful than the conventional Bonferroni methodfor multiple comparions. It controls better the family-wise error rate, i.e. the probability that one or more Type I errors (false positives) will occur (Holm, 1979). The differences in daily preferences (PI/day) for each salt/metal across concentrations was analysed using Generalized Linear Models Estimating (GEE) for Repeated Measures within-subjects.

A factorial model was built to test for the effects of time (days), concentration and the interaction between days and concentration. The effect of mineral salt diets on young worker bees survival was assessed using survival analysis with Cox Regression Models. The proportionality of hazards (PH) assumption, i.e the risk factors affecting time to event (death) are constant over time, was formaly tested for each salt/metal treatment by fitting a univariate Cox Regression Model with time-dependent covariate. If the interaction term (time by covariate (categorical)) did not

reach significance (P> 0.05), the proportionality of hazards assumption prevailed and a standard Cox PH Regression was used. Validating proportionality of hazards indicated that the effect of risk factors did not change with time. Otherwise, Cox Regression with time-dependent covariate was performed. For each salt/metal, the survival rates across concentration levels (risk factor) were compared using contrasts Indicator with control treatment as the reference category. When an effect of concentration was significant (P< 0.05) in the Cox Model, the relative risk (mean Hazard Ratio, HR) was used to express the magnitude of the effect concentration. The HR showed the mean unit increase/decrease of one treatment in the risk of dying in relation to the control treatment (Cox, 1972; Bellera et al., 2010). If HR > 1, the survival of the treatment (e.g. high Na diet) is lower than that of the control treatment (sucrose only, 0 ppm), indicating that the treatment factor presents a high risk.

In cases where treatments had barely an effect on survival with near 100% censored cases by day 6, i.e. bees were still alive at the end of the experiment, Cox Models could not be fitted. Instead, survival curves for each treatment group (e.g. K, Ca, Cu treatments) were acquired using non-parametric Kaplan-Meier estimators (Kaplan and Meier, 1958) to compare factor (concentration) levels. Differences between survival curves were pairwise compared (over strata) by the Log-rank (P< 0.05), Breslow (P< 0.05), and Tarone-Ware (P< 0.05) to account for the whole period of observation. If tests significant, two HR at different time points (day 3 and day 6 – end) were calculated algebrically to express the magnitude of the effect compared to the reference group. For example, on day 3: HR= Survival Probability (control) / Survival Probability (++++ treatment). To compare the effect of mineral identity on young bee survival under high mineral diets, I used the Kaplan-Meier estimator (risk factor: mineral type). Differences between survival curves across treatments were compared using contrasts Indicator (reference group). High salt and high metal diets were tested independently.

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4.5 Results

4.5.1 Preference for Mineral Diets

Using two-choice assays, I found that, in general, young bees exhibited a preference for mineral diets in the lower range of concentrations (Figure 4.4). Both mineral identity and concentration yielded significant effects on total diet preference (Table 4.2). In pollen, salts (Na, K, Ca, Mg) are present at 10x the concentration of metals (Fe, Cu, Zn, Mn); for this reason, I have split the analyses into either salts or metals throughout this Chapter.

When fed salt diets, young bees showed an increased preference for Na at high concentrations. This was demonstrated by PI > 0 (Figure 4.4, sodium), i.e. as the concentration of Na in sucrose solutions increased, preference for Na diets also increased. In addition, bees showed maximum preference for 1,000 Na ppm (++++). Lower concentrations of Na (+, 5 ppm and ++, 50 ppm) did not have a major impact on bee feeding preferences, as bees were neither attracted nor deterred by these diets (PI≈ 0). Similarly, bees did not appear to prefer diets containing K at any of the concentrations tested (10, 100 and 1,000 ppm) (Figure 4.4, potassium). Instead, bees avoided consuming diets high in K (10,000 ppm), Ca (500 ppm) and Mg (3,000 ppm) (Figure 4.4). I further expected that low Ca diets (1 ppm) would be phagostimulatory, though I only found a slight preference for this diet, which did not differ statistically from Ca 50 ppm (+++) (Figure 4.4, calcium). As for Mg diets, at the range of concentrations tested (10, 30, 300, 3,000 ppm), young bees rejected sucrose diets enriched in Mg. Moreover, the highest concentration of Mg(++++, 3,000 ppm) induced the maximum rejection (PI< 0) (Figure 4.4, magnesium)

Table 4.2 Factorial Generalized Linear Models (GzLM) for total diet preference (PI) for each mineral treatment. Analysis of the mean preference of young bees fed salt/metal diets at five levels of concentration as shown in Figure 4.4. Each mineral treatment was measured independently. Salts and metals were analysed separately.

Source	Wald $\chi 2$	df	P value
Salts			
(Intercept)	26.6	1	**<0.001**
salt	113.	3	**<0.001**
concentration	92.2	3	**<0.001**
salt x concentration	142.	9	**<0.001**
Metals			
(Intercept)	19.4	1	**<0.001**
metal	4.41	3	0.22
concentration	542.	3	**<0.001**
metal x concentration	265.	9	**<0.001**

Values in bold highlight a probability value (P value) < 0.05, indicating a mean difference significant at the level of 5%.

Figure 4.4 Feeding preferences of young worker honey bees for mineral liquid diets in two-choice assays.

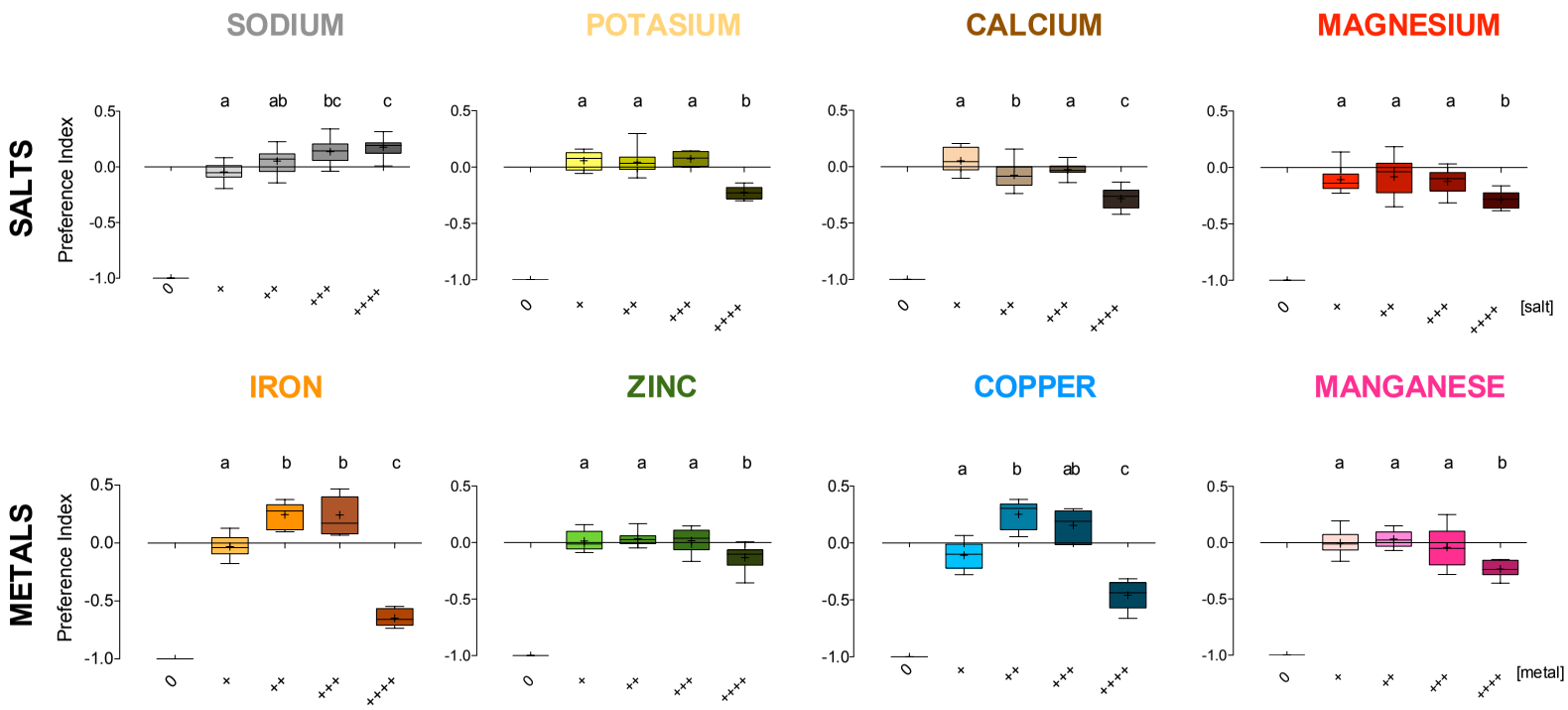


Figure 4.4 Preference index for mineral-laced sucrose diets. Top panel shows honey bee preference indexes (PI) for selected salts (Na, K, Ca, Mg). Bottom panel shows preference indexes for selected metal nutrients (Fe, Zn, Cu, Mn). All mineral nutrients were provided in chloride salts. Each salt or metal was tested at five increasing concentrations ranging from control sucrose (0) up to very high (++++). Concentrations were tailored for each mineral salt, altogether ranging from 0.5 to 10,000 ppm. The concentration range of Na, Ca, Mg and Mn diets did not follow a logarithmic scale (base 10). PI > 0 shows preference for salt or metal diets, whilst PI< 0 indicates preference for sucrose alone in two-choice assays over 6 days. Box-plots indicate the minimum and maximum ranges for each independent treatment; (+) is the mean and (—) the median. Sample size ranges between N= 4—10 box replicates with each containing ~30 honey bees. One-way GzLM was performed to test the effects of concentration for each independent treatment. Salts and metals were analyzed separately. **GzLM: Salts**, Na: χ^2= 40.43, df= 3, P<0.001; K: χ^2= 208.3, df= 3, P<0.001; Ca: χ^2= 81.69., df= 3, P<0.001; Mg: χ^2= 31.61, df= 3, P<0.001. **GzLM: Metals**, Fe: χ^2= 557., df= 3, P<0.001; Zn: χ^2= 22.23, df= 3, P<0.001; Cu: χ^2= 132.6, df= 3, P<0.001; Mn: χ^2= 79.64, df= 3, P<0.001. Differences between groups (treatment concentrations) are denoted by d different letters (pairwise) and considered at 5% (P value <0.05). Control treatment (0 ppm) is artificially depicted as a constant value of PI= -1 (absolute preference for sucrose) only for visual comparison. This treatment was not included in the statistical model.

There was a significant effect of both metal identity and concentration on diet preferences (Table 4.2). When young bees were offered a choice between sucrose alone and metal-enriched sucrose diets < 100 ppm (lower ranges), their response depended on the type of metal tested. All diets high in Fe, Zn, Cu or Mn (500–1,000 ppm) induced rejection. In general, bees fed Fe and Cu diet treatments responded with a similar pattern. I found that of the amount of food bees consumed increased as a function of the concentration of Fe and Cu in the diet (PI for Fe: 1 < 10 < 100 ppm; Cu: 0.5 < 50 < 5 ppm). Maximum metal diet preferences were attained at the medium range of concentrations (Fe: 100 ppm and Cu: 5 ppm) (Figure 4.4, iron and copper). In parallel, diets high in Fe (1,000 ppm) or Cu (500 ppm) were the most deterrent to young bees (Fe: PI= -0.64; Cu: PI= -0.35) compared to high Zn and Mn, and high salt inclusive. As for Zn and Mn diets, within the range of 0.5–50 ppm, bees did not prefer nor reject any of these diets in comparison to sucrose alone. As illustrated in Figure 4.4, pairwise comparisons between lower range diets did not differ significantly. Though, and as expected, high Zn or high Mn diets (both at 500 pm) were significantly avoided by young bees.

4.5.2 Daily Preference for Salt Diets

Young bees' feeding responses in two-choice set-ups were measured over 6 consecutive days. To assess whether and how honey bee mineral diet preferences fluctuate over time, I then tested the effect of days and concentration on the daily preferences (PI/day) for each mineral treatment (Na, K, Ca, Mg, Fe, Cu, Zn, Mn). As shown in Figure 4.5 and Table 4.3, daily preferences for Na and Mg diets significantly depended on both day and concentration (Table 4.3) . I predicted differences in consumption between treatment concentrations, but no major fluctuations were observed over timewithin each treatment. In diets high in Na (1,000 ppm), bees showed a 3-fold increase in preferece for the sodium diet from day 1 to 5, followed by a 1.5-fold decrease at day 6 (Figure 4.5, sodium). Consumption measurements were performed daily and around the same time for each feeding

group (data was controlled for significant outliers). Daily preferences for K and Ca diets were significantly affected by day or concentration as main effects in the model (Table 4.3). As observed for bees fed with the high Mg diets, young bees consistently avoided ingesting high K or Ca diets every day as demonstrated by daily PI < 0 (Figure 4.5, potassium and calcium).

Table 4.3 Factorial Generalized Linear Models (GEE, Repeated Measures, within-subjects: days) testing diet preferences over time (PI/day) for each salt treatment. Analysis of the mean daily preference of young bees fed salt diets at four levels of concentration[1] as shown in Figure 4.5. Each salt treatment was measured independently and analysed separately.

Source: Salts	Wald $\chi 2$	df	P value[2]
Sodium			
(Intercept)	37.7	1	**<0.001**
day	29.0	5	**<0.001**
concentration	40.6	3	**<0.001**
day x concentration	115.	15	**<0.001**
Potassium			
(Intercept)	0.93	1	0.34
day	19.9	5	**<0.001**
concentration	272.	3	**<0.001**
day x concentration	19.6	15	0.19
Calcium			
(Intercept)	46.1	1	**<0.001**
day	23.0	5	**<0.001**
concentration	78.6	3	**<0.001**
day x concentration	16.3	15	0.36
Magnesium			
(Intercept)	107.	1	**<0.001**
day	27.5	5	**<0.001**
concentration	62.7	3	**<0.001**
day x concentration	116.	15	**<0.001**

[1]Sucrose control diet (salt-free) was not included in the analysis. [2]Values in bold highlight a probability value (P value) < 0.05, indicating a mean difference significant at the level of 5%.

Figure 4.5 Patterns of salt feeding and preferences of young worker bees over 6 days.

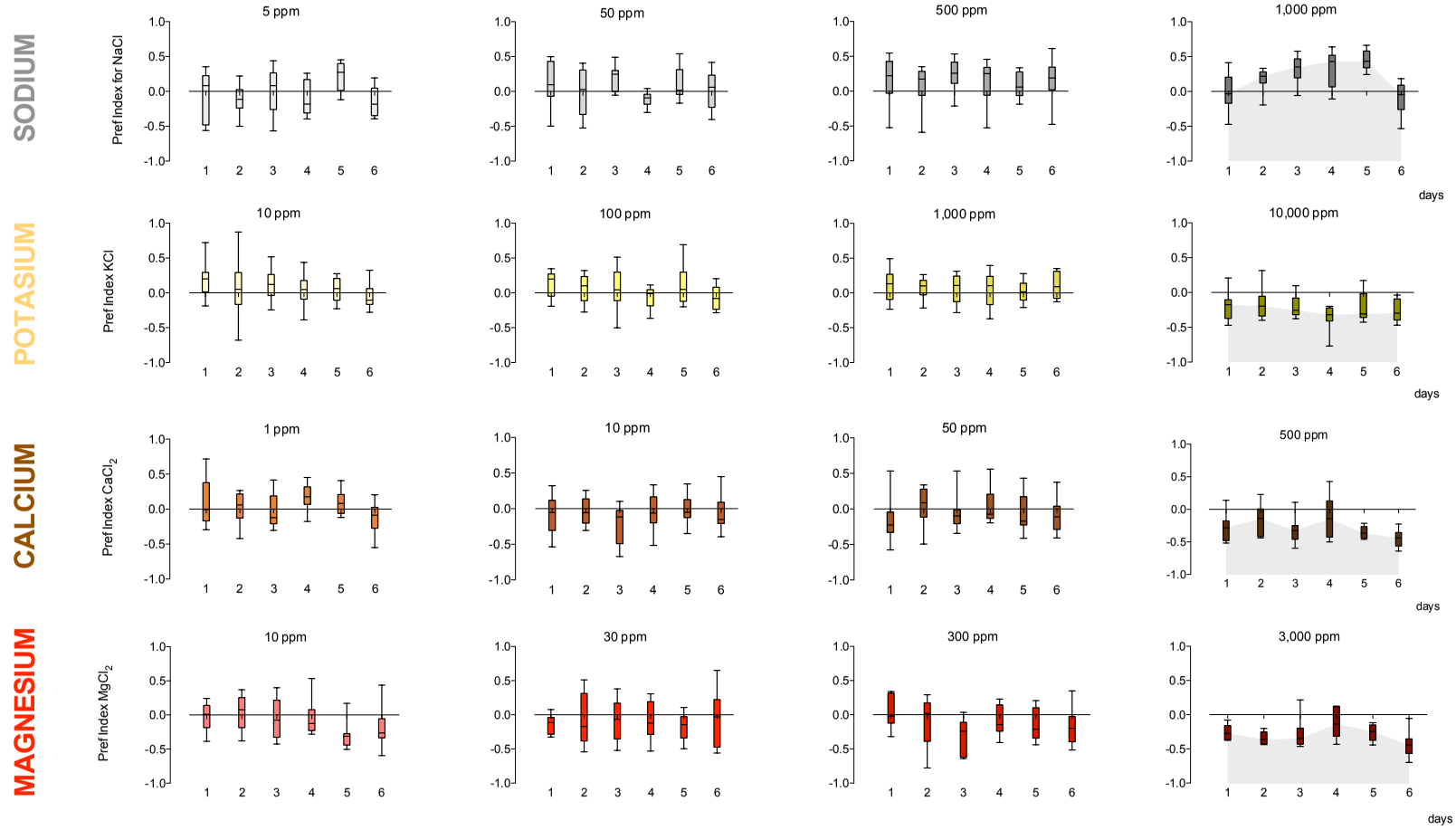


Figure 4.5 Daily feeding preferences of young honey bees fed a choice between sucrose alone and salt-laced sucrose diets over 6 days. Each row represents feeding treatments at increasing concentrations for a single salt overtime. Levels of salt concentrations are in ppm units. Sucrose control treatments (0 ppm) are not shown. Daily preference indexes are plotted for each day. A single panel respects an independent treatment. PI > 0 shows preference for salt diet on that day, whilst PI < 0 indicates preference for sucrose alone. Box-plots indicate the minimum and maximum ranges for each independent treatment; (—) is the median. Each treatment corresponds to N= 10 box replicates each containing ~30 honey bees.

4.5.3 Daily Preference for Metal Diets

Figure 4.6 reveals the daily preferences for metal diets by young honey bees. The young bees' feeding preferences for Fe and Cu diets were a function of both day and concentration (Table 4.4), which indicates that mean preferences over time differed between days within each treatment concentration. Bees fed with the low Fe diets (1 pm), for example, increased/decreased from day 1 to 3, but then did not vary greatly until day 6 (Figure 4.6 , iron). In contrast, bees fed a choice of high Fe or Cu systematically preferred sucrose only diet across days (PI < 0). The concentration of Zn and Mn in diets significantly affected bees' daily preferences (Figure 4.6 and Table 4.4). Bees also rejected diets high in Mn over time, but this was not as clear for bees fed diets high in Zn. Because the Zn treatment was the only one which did not show a clear pattern of rejection of feeding over time (as expected) as there was no significant interaction for days x conc. However, Zn concentration had a significant effect on feeding, (for Zn diets only and excluding control diet (0 ppm), 2-way GEE, concentration: χ^2= 41.9, df= 3, P< 0.001; day: χ^2= 5.39, df= 5, P= 0.37), but pairwise comparisons showed that rejection of high Zn diet was only statistically significant for days 3 and 6.

Table 4.4 Factorial Generalized Linear Models (GEE, Repeated Measures, within-subjects: days) testing diet preferences over time (PI/day) for each metal treatment. Analysis of the mean daily preference of young bees fed metal diets at four levels of concentration[1] as shown in Figure 4.6. Each metal treatment was measured independently and analysed separately.

Source: Metals	Wald $\chi 2$	df	P value[2]
Iron			
(Intercept)	4.89	1	**<0.05**
day	15.2	5	**<0.05**
concentration	599.	3	**<0.001**
day x concentration	123.	15	**<0.001**
Copper			
(Intercept)	1.91	1	0.17
day	21.6	5	**<0.001**
concentration	125.	3	**<0.001**
day x concentration	75.3	15	**<0.001**
Zinc			
(Intercept)	3.05	1	0.08
day	5.66	5	0.34
concentration	41.9	3	**<0.001**
day x concentration	13.6	15	0.55
Manganese			
(Intercept)	14.2	1	**<0.001**
day	7.41	5	0.19
concentration	75.9	3	**<0.001**
day x concentration	24.6	15	0.06

[1]Sucrose control diet (metal-free) was not included in the analysis. [2]Values in bold highlight a probability value (P value) < 0.05, indicating a mean difference significant at the level of 5%.

Figure 4.6 Patterns of metal feeding and preferences of young worker bees over 6 days.

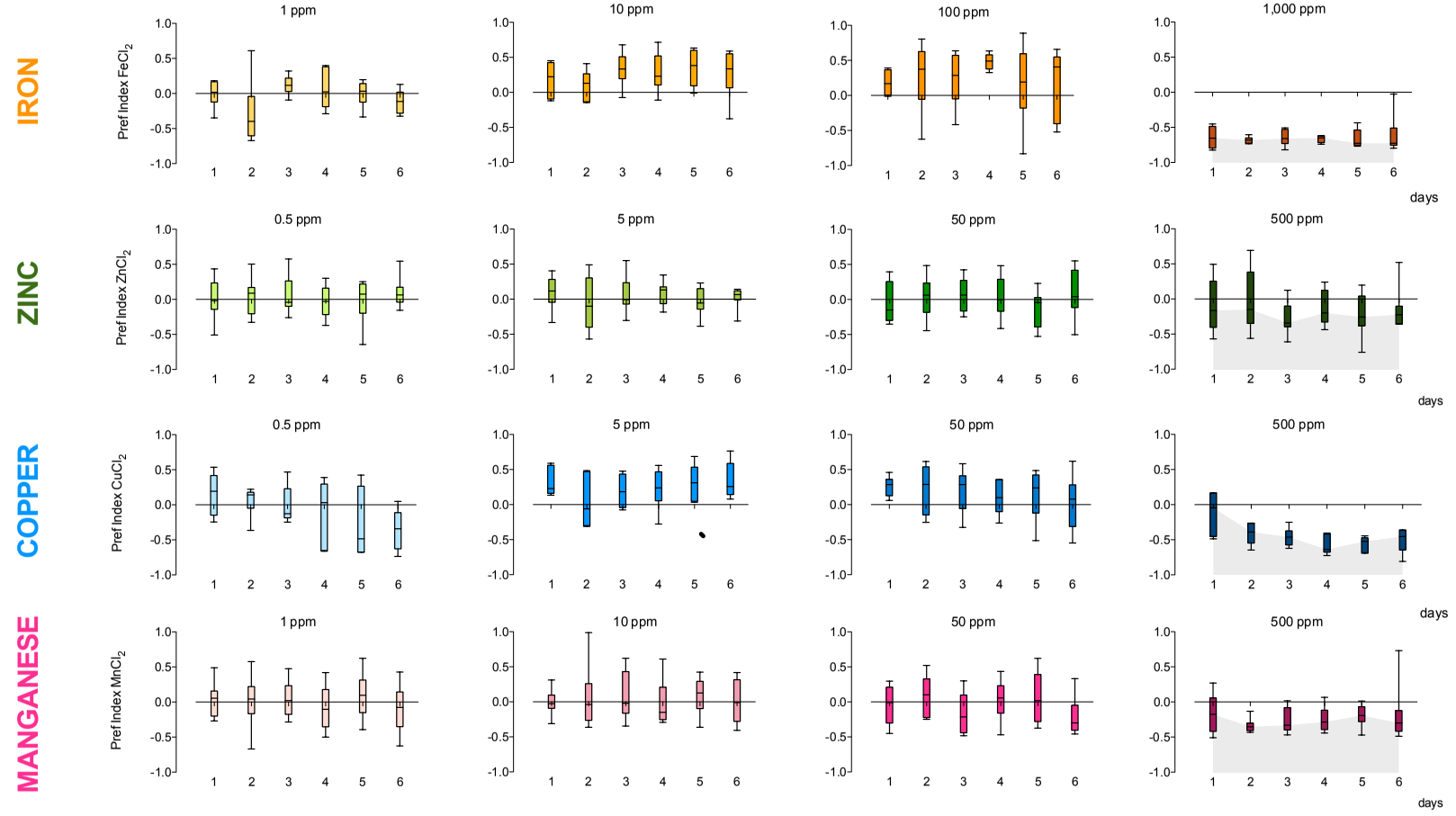


Figure 4.6 Daily feeding preferences of young honey bees fed a choice between sucrose alone and metal-laced sucrose diets over 6 days. Each row represents feeding treatments at increasing concentrations for a single metal overtime. Levels of metal concentrations are in ppm units. Sucrose control treatments (0 ppm) are not shown. Daily preference indexes are plotted for each day. A single panel respects an independent treatment. PI > 0 shows preference for metal diet on that day, whilst PI < 0 indicates preference for sucrose alone.Box-plots indicate the minimum and maximum ranges for each independent treatment; (—) is the median. Each treatment corresponds to N= 6—10 box replicates each containing ~30 honey bees.

4.5.4 Effects of salt diets in the feeding responses of young worker bees

I also explored whether bees regulated their diet intake around a specific quantity of salt/metal diet over the course of 6 days. To do this, I measured the volume consumed and the total volume of all solutions consumed. I considered total sucrose solution consumed by control treatment' bees (salt/metal-free treatments, 0 ppm) as the standard reference. Results for salt treatments are indicated in Figure 4.7 Salt diet consumption (volume) was signifcantly affected by both salt identity and concentration (Table 4.5). This outcome was anticipated given the data shown in Table 4.5 (salts). Young bees consistently consumed more of Na diets as concentration increased (Figure 4.7, Salt Diet: sodium). Interestingly, I found that Na diets produced a significant increase in the total volume consumed per bee (e.g. Na, 500 ppm: 58.5 μL/bee) compared to control treatments (Na, 0 ppm: 47.8 μL/bee) (Figure 4.7, Total Diet: Sodium). Bees given a choice of K diets, ingested similar volumes of each (10, 100 and 1,000 ppm), with the exception of bees fed 10,000 K ppm diets, which ate less. Nevertheless, total diet consumption was not affected by K concentration (Figure 4.7, Total Diet: potassium). Likewise, bees fed high Ca and Mg diets ate significantly less food. Bees under high Ca or Mg diets reported the minimum volumes consumed per bee (Ca: 18.8 μL/bee; Mg: 19.7 μL/bee) compared to lower concentrations (Figure 4.7, Salt Diet: calcium and magnesium). I expected a reduction on the feeding responses of bees fed high Ca treatments, but total diet consumption was not statistically different from the control (Ca, 0 ppm: 61.3 μL/bee) (Figure 4.7, Total Diet: calcium). Diet consumption in sucrose control treatment was highly variable compared to other Ca treatments (52.4 to 73.1 μL/bee). Bees fed the low Mg treatments (10 and 30 ppm) consumed greater volumes of total diet contrasting to control bees (Figure 4.7, Total Diet: magnesium). In spite of the fact that the bees rejected and consumed less of high Mg diet (++++, 3,000 ppm) (Figure 4.4 and 4.7, Salt Diet: magnesium), the total volume ingested in this diet was similar to total volumes consumed by control bees (Figure 4.7, Total Diet: magnesium).

Table 4.5 Factorial Generalized Linear Models (GzLM) testing the effect of salt and concentration on young workers feeding responses in two-choice feeding assays over 6 days. Analysis of diet volume consumption (μL/bee) as shown in Figure 4.7. Each salt treatment was measured independently.

Source: Salts	Wald $\chi 2$	df	P value
Salt Diet			
(Intercept)	7884.	1	**<0.001**
salt	80.9	3	**<0.001**
concentration	8371.	4	**<0.001**
salt x concentration	179.	12	**<0.001**
Total Diet			
(Intercept)	24105.	1	**<0.001**
salt	26.9	3	**<0.001**
concentration	3.64	4	0.46
salt x concentration	88.5	12	**<0.001**

Values in bold highlight a probability value (P value) < 0.05, indicating a mean difference significant at the level of 5%.

Figure 4.7 Total volume consumption by young worker bees in salt treatments.

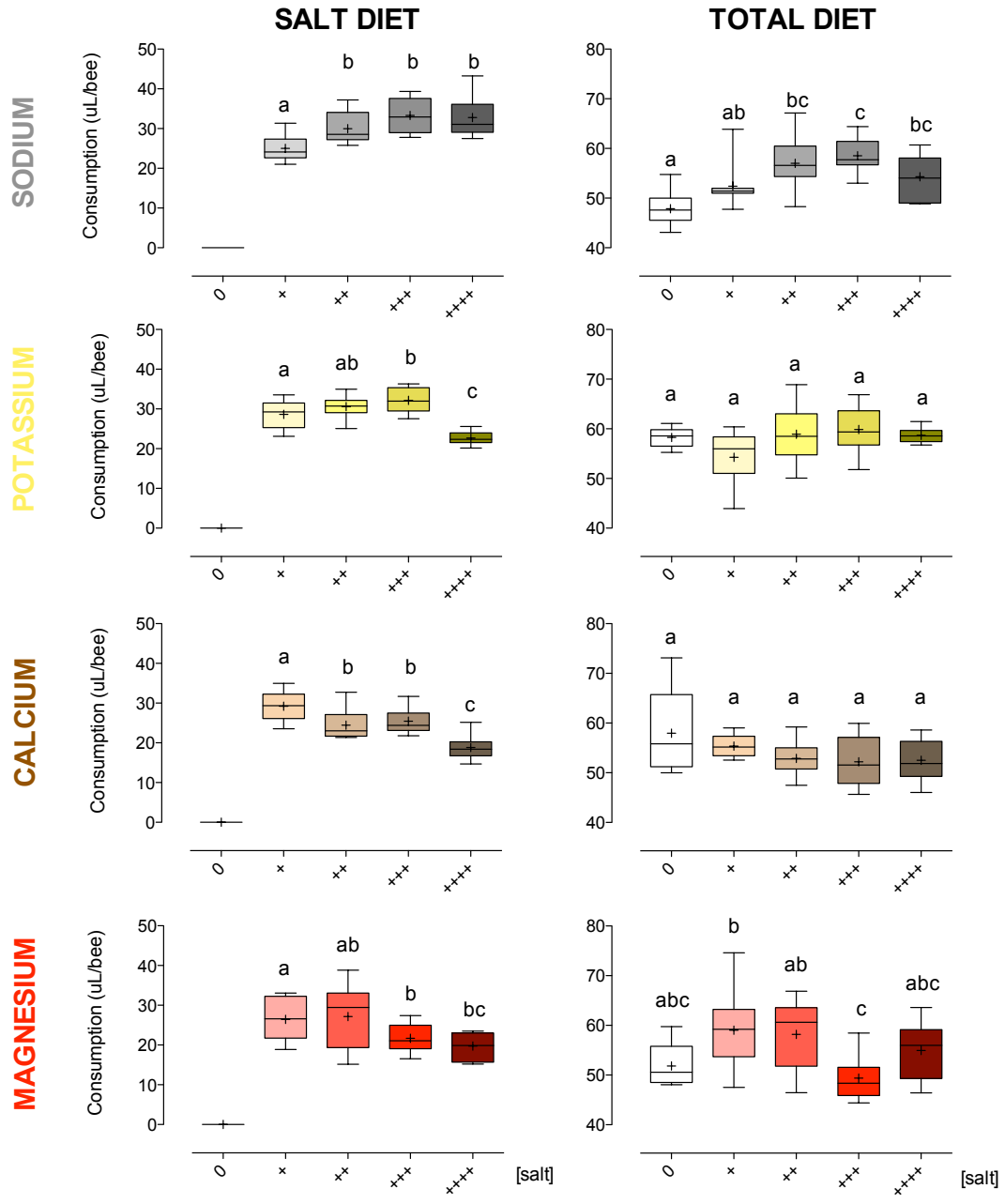


Figure 4.7 Volume consumption in salt feeding treatments by young worker bees under two-choice assays over 6 days. Four salts were tested (Na, K, Ca, Mg) at five levels of concentration ranging from control sucrose (0) up to very high (+++ +). Concentrations were tailored for each salt and only K treatment followed a logarithmic scale (base 10). Treatment denoted as 0 corresponds to control treatment (salt-free; sucrose only). Each row depicts data for one salt (N= 5—10 boxes). Left-side panels depict the mean volume consumed per salt diet (µL/bee). Sucrose control treatment (0 ppm) is shown only for visual reference. Right-side panels show total volume consumed per treatment. Box-plots indicate the minimum and maximum ranges for each independent treatment; (+) mean and (—) median. One-way GzLM was performed to test the effects of concentration for each independent treatment (see S14 Table, Appendix A). Differences between groups (treatment concentrations) are denoted by different letters (pairwise) and considered at 5% (P value <0.05).

4.5.5 Effects of metal diets in the feeding responses of young worker bees

The results for the metal diet treatments are indicated in Figure 4.8. The magnitude of metal diet consumption (volume) depended on both metal identity and concentration (Table 4.6). Consumption of Fe diets revealed a distinctive profile in contrast to Cu, Zn and Mn diets. Young bees ingested greater volumes of Fe diets as a function of concentration (Fe: 1 to 100 ppm). Bees fed Fe 10 and 100 ppm diets ingested significantly higher volumes than Fe 1 and 1,000 ppm diets (Figure 4.8, Metal Diets: iron). As expected, high Fe (1,000 ppm) were consumed very little (8.89 μL/bee). For the Cu and Mn diets, no major differences were observed between the volumes ingested from lower range diets (Cu: 0.5, 5 and 50 ppm; Mn: 1, 10 and 50 ppm). Instead, only bees fed high Cu and high Mn diets (500 ppm) consumed significantly less of the diet solution (Figure 4.8, Metal Diets: copper and manganese). Additionally, neither Cu nor Mn treatments affected the total volume of diet consumed. The observed feeding responses for Zn treatments as demonstrated by pairwise comparisons in Figure 4.8 were not a smooth function of concentration The volume of diet ingested (0.5, 5, 50 and 500 ppm) oscillated across concentrations. This was also the case for total diet consumed in Zn treatments (Figure 4.8, Total Diet: zinc). The total diet consumed by control bees (Zn, 0 ppm) was comparable to total volumes consumed by bees fed Zn 5 and 500 ppm, but not Zn 0.5 and 50 ppm diets. However, it is worth noting that the sucrose control diet consumption also varied across the mineral salt treatments, perhaps indicating that other variables were influencing food consumption in this portion of the experiments (S13 Figure, Appendix A). Overall, bees assigned to Cu treatments consumed the least volume of diet, including control diets, when compared to total volumes ingested in Na, K, Ca, Mg, Fe, Zn and Mn treatments: 0 ppm (μL/bee): Mn (67.0) > Ca (61.3) > Zn (60.0) > K (58.3) > Fe (55.8) > Mg (51.8) > Na (47.8) > Cu (43.3) (Figure 4.8, Total Diet).

Table 4.6 Factorial Generalized Linear Models (GzLM) testing the effect of metal and concentration on young workers feeding responses in two-choice assays over 6 days. Analysis of diet volume consumption (μL/bee) as shown in Figure 4.8. Each metal treatment was measured independently.

Source: Metals	Wald $\chi 2$	df	P value
Metal Diet			
(Intercept)	5187.	1	**<0.001**
metal	313.	3	**<0.001**
concentration	5684.	4	**<0.001**
metal x concentration	123.	12	**<0.001**
Total Diet			
(Intercept)	11153.	1	**<0.001**
metal	658.	3	**<0.001**
concentration	114.	4	**<0.001**
metal x concentration	268.	12	**<0.001**

Values in bold highlight a probability value (P value) < 0.05, indicating a mean difference significant at the level of 5%.

Figure 4.8 Total volume consumption by young worker bees in metal treatments

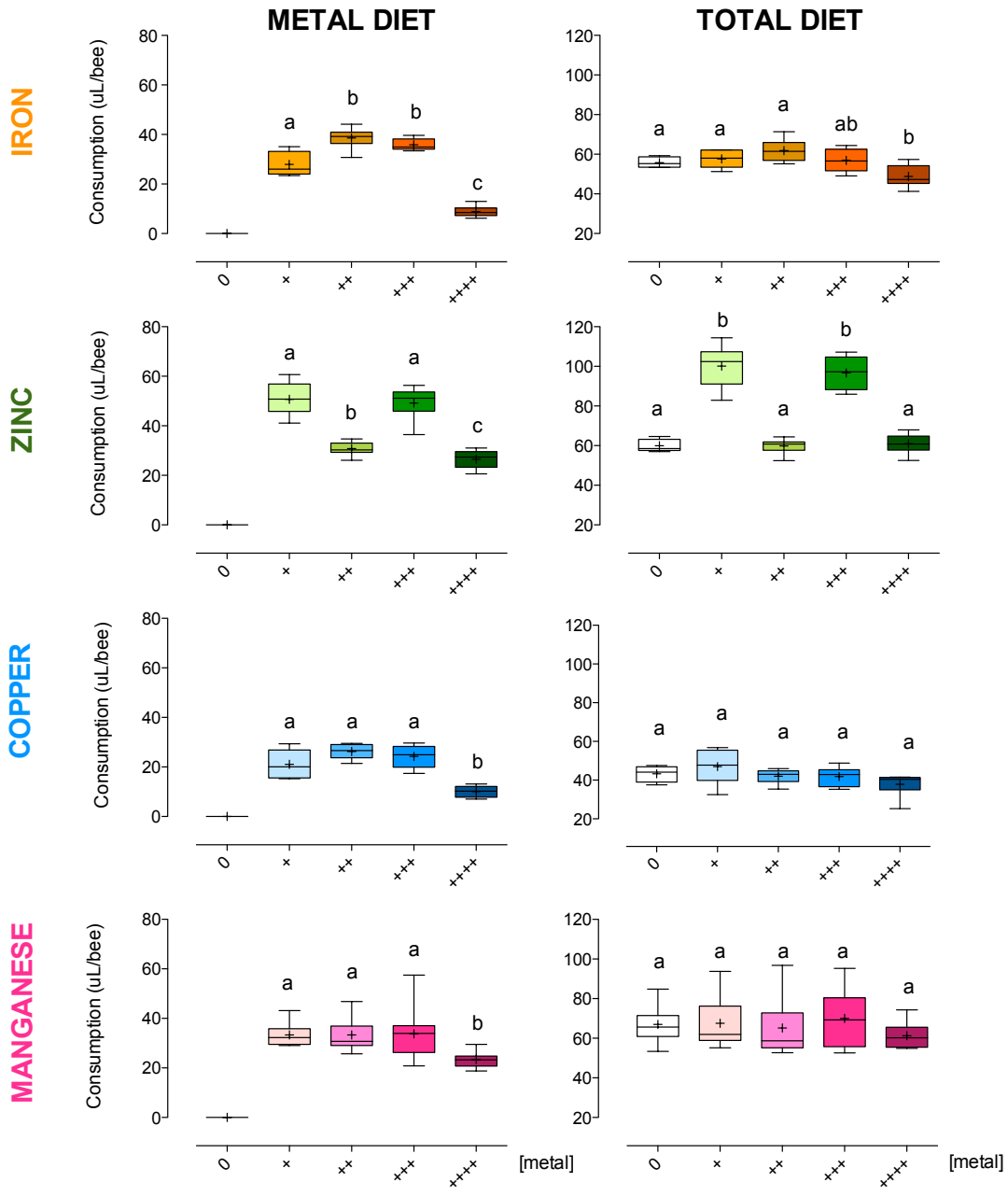


Figure 4.8 Volume consumption in metal feeding treatments by young worker bees under two-choice assays over 6 days. Four metals were tested (Fe, Zn, Cu, Mn) at five levels of concentration ranging from control sucrose (0) up to very high (+ +++). Concentrations were tailored for each metal and only Mn treatment did not follow a logarithmic scale (base 10). Treatment denoted as 0 corresponds to control treatment (salt-free; sucrose only). Each row depicts data for one metal (N= 6—10 boxes). Left-side panels depict the mean volume consumed per metal diet (µL/bee). Sucrose control treatment (0 ppm) is shown only for visual reference. Right-side panels show total volume consumed per treatment. Box-plots indicate the minimum and maximum ranges for each independent treatment; (+) mean and (—) median. One-way GzLM was performed to test the effects of concentration for each independent treatment (see S14 Table, Appendix A). Differences between groups (treatment concentrations) are denoted by different letters (pairwise) and considered at 5% (P value <0.05).

4.5.6 Effects of mineral diets in water consumption

Distilled water was available throghout the feeding experiments. Results for water consumption were analysed independently and are reported in Figure 4.9. Water consumption within each mineral group was not expected to change. However, it varied as a function of mineral type and concentration (GzLM: mineral x concentration: χ^2= 131., df= 28, P<0.001; mineral: χ^2= 2,453., df= 7, P<0.001; concentration: χ^2= 6.97, df= 4, P> 0.05. Water consumption was not affected in five mineral treatments (Na, K, Ca, Mg and Mn), though water ingestion significantly increased with Fe and Cu treatments (concentration for Fe: χ^2= 95.9, df= 4, P< 0.001; Zn: χ^2= 61.8, df= 4, P< 0.001; Cu: χ^2= 20.6, df= 4, P< 0.001; Na: χ^2= 9.46, df= 4, P= 0.05; Ca: χ^2= 6.87, df= 4, P= 0.14; Mn: χ^2= 5.16, df= 4, P= 0.27; K: χ^2= 4.60, df= 4, P= 0.33; Mg: χ^2= 4.51, df= 4, P= 0.34).

Figure 4.9 Water consumed by young bees in choice feeding assays testing mineral diets preference and intake.

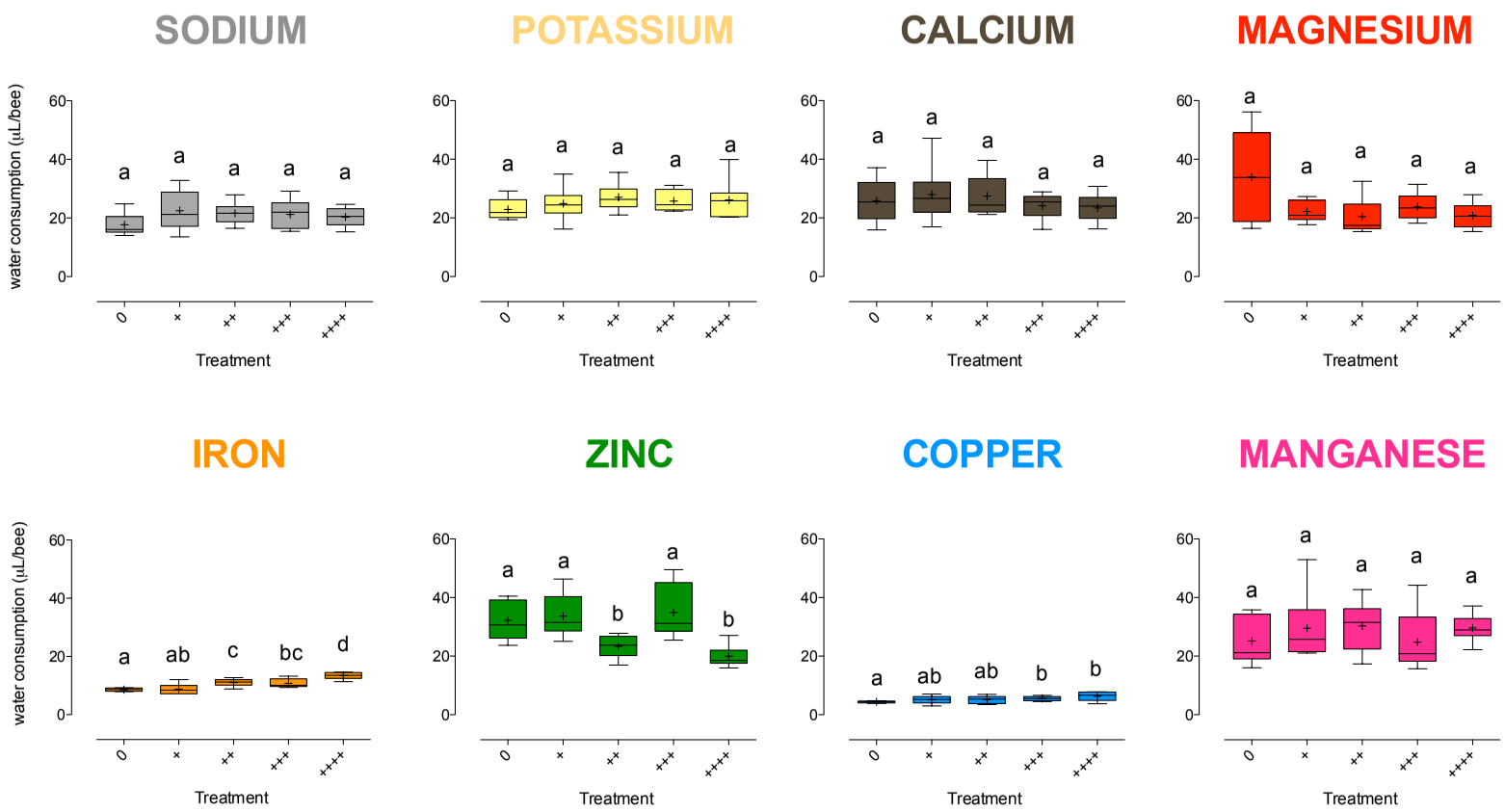


Figure 4.9 Water consumption in choice feeding cohorts of young honey bees. Top and bottom panel of graphs represent salt and metal feeding assays, respectively. Each salt or metal treatment ranged from no salt diet (0 ppm) up to high salt/metal treatment (++++). Each treatment included N= 4—10 box replicates allocating ~30 bees. Each box presented bees with two feeding tubes of distilled water, two tubes of control solution (sucrose 1.0 M alone) and two tubes of testing solution (sucrose laced with one concentration of one salt/metal). Here, only water consumption is presented and was analyzed independently. GzLM: mineral x concentration: χ^2= 131., df= 28, P<0.001; mineral: χ^2= 2,453., df= 7, P<0.001; concentration: χ^2= 6.97, df= 4, P> 0.05. Different letters indicate differences between group means at 5% (P< 0.05).

4.5.7 Effects of short-term mineral feeding on the fresh body weight of young adult bees

Finally, at the end of each the fresh body weight of young bees (N≈50/treatment concentration) was measured to test the effect of mineral diets on the total weight. Overall, there was a significant effect of both mineral type and treatment concentration on the mean body fresh weight at day 6 (GzLM: mineral x concentration χ^2= 119., df= 28, P< 0.001; mineral: χ^2= 306., df= 7, P<0.001; concentration: χ^2= 10.8, df= 4, P< 0.05). Each salt/metal concentration had a significant impact on fresh body weights of bees in Na, K, Zn and Cu treatments, but not in Ca, Mg, Fe and Mn (concentration for Na: χ^2= 38.1, df= 4, P< 0.001; K: χ^2= 35.9, df= 4, P< 0.001; Zn: χ^2= 23.2, df= 4, P< 0.001; Cu: χ^2= 15.6, df= 4, P< 0.01; Ca: χ^2= 5.84, df= 4, P= 0.21; Mg: χ^2= 5.77, df= 4, P= 0.22; Fe: χ^2= 4.71, df= 4, P= 0.32; Mn: χ^2= 6.70, df= 4, P= 0.15) (Figure 4.10).

Figure 4.10 Fresh body weight of young honey bees fed mineral diets in two-choice assays over 6 days.

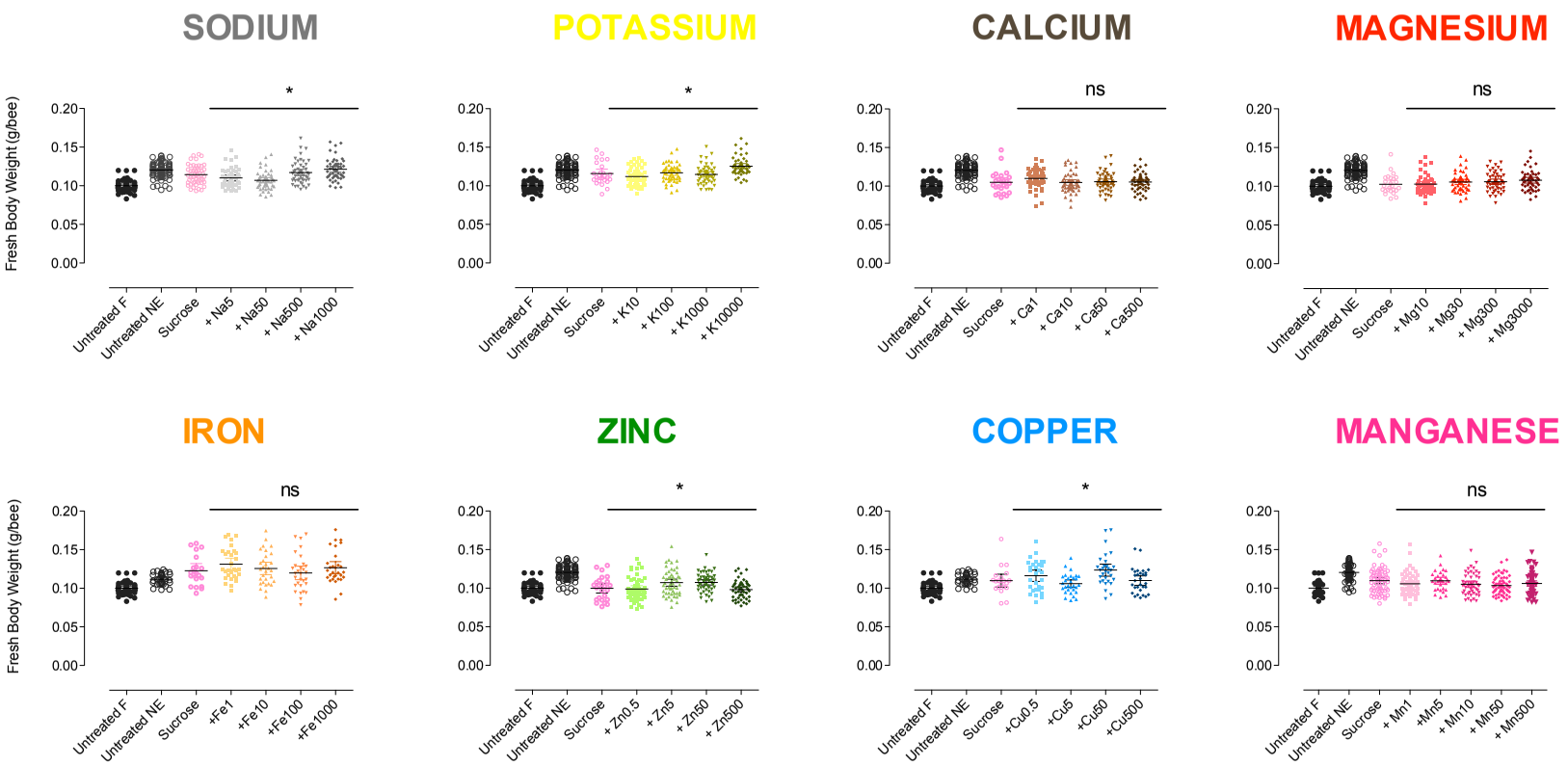


Figure 4.10 Fresh body weight of worker honey after 6 days of feeding in two-choice assays. Sucrose diets (1.0 M) laced with single minerals were tested at five concentrations. Control diet was sucrose (1.0 M) only solutions (pink data points). At day 6, N= 5 bees/box were weighed (N=25-50/treatment). For comparison, forager bees (untreated F, N= 50) and newly emerged bees (untreated NE, N= 89) were also weighed. Untreated bees (no feeding) were not included in the statistical model. Scatter plots depict the mean ± 95% CI. GzLM: mineral x treatment χ^2= 119., df= 28, P< 0.001; mineral: χ^2= 306., df= 7, P< 0.001; treatment: χ^2= 10.8, df= 4, P<0.05. (*) indicates statistical significance at 5% between groups and (ns) denotes groups are not significantly different (P > 0.05).

4.5.8 Effects of salt feeding on the survival of young worker honey bees

To examine the risks of feeding on mineral salt diets varying in concentartion over 6 days, the survival of young worker bees was assessed with Cox Models and Kaplan Meier (KM) survival analyses. Mean survivorship curves for the different treatments over time are presented in Figure 4.11. Mineral salt feeding at this range of concentrations (0.5–10,000 ppm) did not have a negative impact on the survival of young workers over 6 days. In a choice situation, five (Na, K, Ca, Fe and Mn) out of eight feeding treatments, regardless of concentration, revealed that > 95% of bees were still alive at day 6 (Figure 4.11 a, b, c, f, i; see also S15 Table, Survival Table, Appendix A). Otherwise, only three treatments (Mg, Zn and Cu) affected significantly bee survival (Figure 4.11 d, g, h). The Cox Regression Models were used to test the risk effect of concentration on bee survival for each Na, Mg, Fe, Zn and Mn treatment. The proportionality of hazards assumption, i.e. the risk factors affecting time to event (death) are constant over time, was validated for all treatments (time x conc, S16 Table, Appendix A). Only K, Ca and Cu results could not be fitted with Cox Models due to the high proportion of right-censored data, i.e. the study ended before any event (death) had occurred (see S15 Table, Survival Table, Appendix A). Therefore, the survival probability for K, Ca and Cu treatments was estimated with the KM method instead.

Na (5, 50, 500, 1,000 ppm), K (10, 100, 1,000, 10,000 ppm) and Ca (1, 10, 50, 500 ppm) treatments did not show a significant impact on young bee survival (Figure 4.11 a, b, c, d; Table 4.7) compared to control treatments. Bees provided with high Na diets (++++, 1,000 ppm) showed high survival rates at day 6, akin to control bees fed sucrose only diets (survival rates: control, 96.9%, N= 322; Na 1,000, 99.3%, N= 301; see also Figure 4.11 a and Table 4.7). Likewise, K diets as high as 10,000 ppm (1% w/v) did not increase the risk of death compared to control treatments. I found no significant differences between survival curves at any K concentration over the whole period of observation (KM tests: Log-Rank: χ^2= 3.51, df= 4, P= 0.48; Breslow: χ^2= 3.52, df= 4, P= 0.48; Tarone-Ware: χ^2= 3.51, df= 4, P= 0.48) (Figure 4.11 b). More

than 98.0% of bees were still alive at day 6 in K treatments (S15 Table, Survival Table, Appendix A). As for Ca treatments, similar high survival rates (> 99.0%) were also found across concentrations with no significant differences (KM tests: Log-Rank: χ^2= 4.85, df= 4, P= 0.30; Breslow: χ^2= 4.86, df= 4, P= 0.30; Tarone-Ware: χ^2= 4.86, df= 4, P= 0.30) (Figure 4.11 c). On the contrary, Mg diet treatments (10, 30, 300, 3,000 ppm) influenced significantly the survival of young bees (Figure 4.11 d). Pairwise differences between the mean survival curves for Mg treatments are shown in Table 4.7. Bees ingesting high Mg diets (++++, 3,000 ppm) had a 5.17 times risk increase of dying compared to sucrose alone (survival rates: control, 98.0%, N= 148; Mg 3,000, 86.8%, N= 305).

Table 4.7 Univariate Cox PH Regression Models testing the effect of salt feeding treatments on the survival of young worker bees in two-choice assays conducted over 6 consecutive days. Each salt treatment was measured independently for the effects of salt concentration as shown in Figure 4.11 (a and d). Control treatments included sucrose only diets.

Source: Salts	Risk Factor	Wald $\chi 2$	P value	Exp(ß)‡‡	95% CI for Exp(ß)	
					Lower	Upper
Sodium‡	Concentration	8.59	0.07			
	0[1]					
	+	<0.01	0.98	0.99	0.41	2.38
	++	2.33	0.13	0.41	0.13	1.29
	+++	3.26	0.07	0.31	0.08	1.11
	++++	4.00	**0.05**	0.21	0.05	0.97
Magnesium‡	Concentration	25.2	**< 0.001**			
	0[1]					
	+	2.06	0.15	2.48	0.72	8.57
	++	1.52	0.22	0.33	0.05	1.94
	+++	1.16	0.28	2.00	0.57	7.10
	++++	7.39	**0.01**	5.17	1.58	16.9

‡ Only sodium and magnesium data could be fitted in Cox Regression Models. ‡‡Exp(ß) refers to the relative risk (mean HR). Potassium and calcium data results were analysed with KM estimators as described in the main text. [1]Contrasts Indicator for each treatment (Reference Category). Values in bold highlight a probability value (P value) < 0.05, indicating a mean significant difference at the level of 5%.

4.5.9 Effects of metal feeding on the survival of young worker honey bees

The results revealing the effects of metal diet treatments on young bee survival are indicated in Figure 4.11 (f, g, h, i, j). Fe and Mn treatments did not increase the risk of dying in young bees under 1, 10, 100, 1,000 Fe ppm nor 1, 10, 50, 500 Mn ppm treatments during their first 1–7 days of adulthood. The analyses indicated that for both metal treatments (Fe or Mn) there was no significant effect of concentration on bee survival (Table 4.8). Therefore, bees treated with either Fe or Mn diets demonstrated lower risks of dying and as those observed for sucrose only-fed bees (Figure 4.11 f, i). Zn and Cu treatments, in contrast to Fe and Mn, significantly affected young bee survival depending on the concentration (Figure 4.11 g, h). The presence of high Zn (++++, 500 ppm) in sucrose solutions suggested a 4.86 increase in the hazard when compared to Zn-free diets (survival rates: control, 97.9%, N= 146; Zn 500, 90.3%, N= 310) (Table 4.8).

Figure 4.11 Risk of dying of young worker bees fed mineral diets in two-choice feeding assays over 6 days.

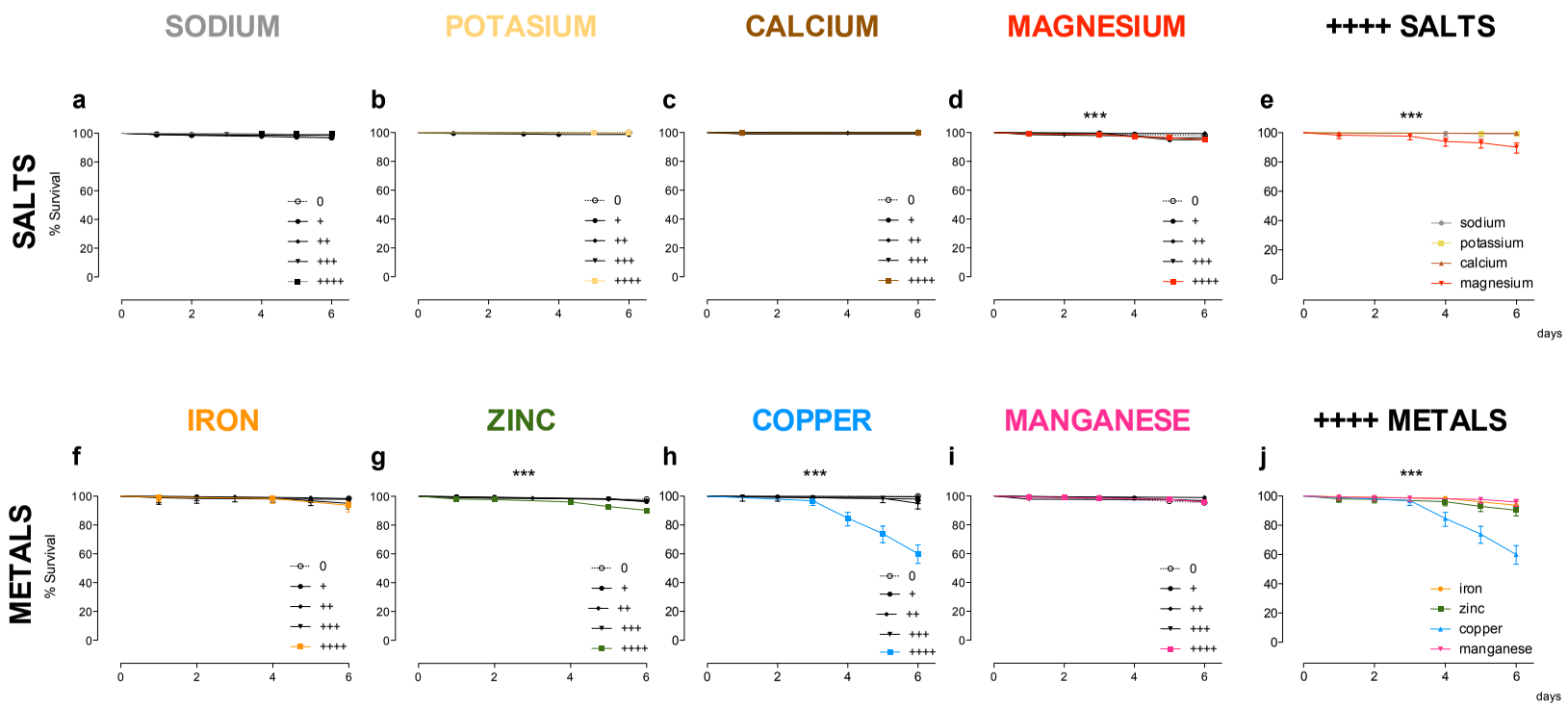


Figure 4.11 Survivorship data for young worker bees fed a choice between sucrose (1.0 M) and mineral-enriched sucrose solutions over 6 consecutive days. Feeding assays were conducted over the spring/summer season (May and August). **Panels (a, b, c, d, e)** depict survival % of bees in salt treatments. **Panels (f, g, h, i, j)** show the survival % of bees under metal treatments. All minerals were chloride-conjugated compounds. Concentrations were tailored for each mineral salt, altogether ranging from 0.5 to 10,000 ppm. Control treatments consisted of 1.0 M sucrose only diet (0). The concentration range of Na, Ca, Mg and Mn diets did not follow a logarithmic scale (base 10). The survival curve respecting the highest concentration (++++) of each salt/metal is highlighted according to the colour code. **Panels e** (salts) and **j** (metals) represent the survival curves at the highest concentrations (++++). Error bars indicate the 95% CI for each independent treatment. Each treatment corresponds to N= 4—10 box replicates for control treatments and N= 5—10 boxes for mineral treatments, each box containing ~30 young bees. Data were analysed using the Cox Regression Model (Na, Mg, Fe, Zn, Mn, ++++ Salts and ++++Metals) or Kaplan-Meier estimator (K, Ca and Cu). (***) Indicate overall statistical significance for concentration at the level of 5% ($P < 0.05$) (also see S15 and S16 Tables, Appendix A).

Table 4.8 Univariate Cox PH Regression Models testing the effect of metal feeding treatments on the survival of young worker bees in two-choice assays conducted over 6 consecutive days. Each metal treatment was analysed independently for the effects of metal concentration as shown in Figure 4.11 (f, g, i). Control treatments included sucrose only diets.

Source: Metals	Risk Factor	Wald $\chi 2$	P value	Exp(ß)‡‡	95% CI for Exp(ß)	
					Lower	Upper
Iron‡	Concentration	8.62	0.07			
	0[1]					
	+	2.21	0.14	3.14	0.70	14.2
	++	<0.01	0.96	1.05	0.18	6.26
	+++	0.12	0.74	1.33	0.24	7.25
	++++	3.24	0.07	3.96	0.89	17.7
Zinc‡	Concentration	20.0	**< 0.001**			
	0[1]					
	+	0.76	0.38	1.76	0.49	6.32
	++	0.29	0.59	1.43	0.39	5.28
	+++	0.80	0.37	1.79	0.50	6.42
	++++	6.81	**0.01**	4.86	1.48	15.9
Manganese‡	Concentration	5.88	0.21			
	0[1]					
	+	0.77	0.38	0.68	0.29	1.60
	++	5.43	**0.02**	0.23	0.06	0.79
	+++	0.15	0.70	0.86	0.38	1.91
	++++	0.09	0.77	0.89	0.41	1.95

‡ Only iron, zinc and manganese data could be fitted in Cox Regression Models. ‡‡Exp(ß) refers to the relative risk (mean HR). Copper data results were analysed with KM estimators as described in the main text. [1]Contrasts Indicator for each treatment (Reference Category). Values in bold highlight a probability value (P value) < 0.05, indicating a mean significant difference at the level of 5%.

Survival curves for high Cu diets (++++, 500 ppm) were statistically different from control diets as revealed by KM statistics (KM tests: Log-Rank: χ^2= 73.4, df= 4, P< 0.001; Breslow: χ^2= 72.3, df= 4, P< 0.001; Tarone-Ware: χ^2= 72.9, df= 4, P< 0.001) (Figure 4.11 h). The hazard of dying under high Cu compared to control diets increased over time from 1.05 and then 1.79-fold, this is from day 3 to 6, respectively. I next investigated further the effect of salt or metal identity on the survival probability of young bees. Because, the lower ranges of concentration did not differ largely from control treatments, only the highest concentrations of salt (Na, K, Ca, Mg) or metal (Fe, Zn, Cu, Mn) treatments were analysed. The survival curves for the four salts tested were statistically different, with bees fed high Mg (++++, 3,000 ppm)

presenting 15.3 times higher risk of dying than in high Na (++++, 1,000 ppm), for example (Table 4.9, ++++Salts). In decreasing order, the mean survival rates for high salt diets were K = Ca (99.7%) > Na (99.3%) > Mg (86.8%). Amongst the high metal group, I found that metal type had a significant impact on the risk of dying. Bees ingesting high Cu diets (500 ppm) had the highest mortality. Compared to high Mn diets (500 ppm), young bees treated with high Cu had a 11.8x increase in the relative risk of dying after 6 days in choice feeding cohorts (Figure 4.11 j and Table 4.9). In decreasing order, the mean survival rates for high metal diets were Mn (95.9%) > Fe (93.7%) > Zn (90.3%) > Cu (58.7%).

The survival curves of control treatments (sucrose only) across mineral salt treatments (factor) were tested with KM estimators (S13 Figure c, Appendix A). For the whole period of observation, statistical differences were reported (KM tests for mineral type: Log-Rank: χ^2= 19.5, df= 7, P< 0.001; Breslow: χ^2= 19.4, df= 7, P< 0.001; Tarone-Ware: χ^2= 19.5, df= 7, P< 0.001). In decreasing order, the survival probabilities for control treatment across salt/metal groups were K = Ca = Cu (100%) > Fe (98.4%) > Mg (98.0%) > Zn (97.9%) > Na (96.6%) > Mn (95.4%). Survival rates of control bees in Mn group treatment were statistically different from control bees in K, Ca and Fe group treatments (P< 0.05). In Na assays, survival rates of control bees were statistically different from those in K, Ca and Cu assays. Whereas, sucrose only-treated bees in Mg, Fe and Zn assays showed survivorships similar to all the remaining groups.

Table 4.9 Univariate Cox PH Regression Models testing the effects of high (++++) salt or metal diets on the survival of young worker bees maintained in two-choice feeding cohorts over 6 consecutive days as shown in Figure 4.11 (e and j). Salts and metals were analysed independently. Comparisons between mineral salt within each group are indicated.

Source	Risk Factor	Wald $\chi 2$	P value	Exp(ß)‡	95% CI for Exp(ß)	
					Lower	Upper
++++Salts	Salt Type	34.1	**< 0.001**			
	Sodium[1]					
	Potassium	0.33	0.57	0.49	0.05	5.45
	Calcium	0.35	0.55	0.48	0.04	5.32
	Magnesium	13.9	**< 0.001**	15.3	3.65	63.9
++++Metals	Metal Type	126.	**< 0.001**			
	Iron	1.16	0.28	1.55	0.70	3.46
	Zinc	6.89	**0.01**	2.45	1.26	4.79
	Copper	64.6	**< 0.001**	11.8	6.46	21.5
	Manganese[1]					

[1]Contrasts Indicator for each group (Reference Category). ‡Exp(ß) refers to the relative risk (mean HR). Values in bold highlight a probability value (P value) < 0.05, indicating a mean significant difference at the level of 5%.

4.6 Discussion

The results of this study support the hypothesis that young adult bees not only found palatable minerals in dietary solutions, but also adjusted their behaviour by shifting feeding patterns to regulate mineral intake. In the context of choice cohorts, by measuring how much young bees ate between the two diets for each mineral treatment, I report four important and novel findings: 1) young bees not tending larvae nor the queen perceived and selected specific minerals in food; 2) not all minerals acted as phagostimulants at low levels, but were deterrent at sufficiently high levels; 3) young bees showed behavioural regulation of mineral intake, but not all minerals are regulated in the same extent; and 4) different minerals evoked different gustatory responses. The Bertrand's rule was proven reliable as a framework to predict optimal intake and feeding preference thresholds of certain mineral nutrients (refer to Figure 1.4, Chapter 1 and results in Figure 4.4, Fe and Cu).

4.6.1 *Free-flying young adult honey bees self-select mineral diets*

Free-flying young bees demonstrated dietary self-selection of individual minerals in a concentration-dependent choice pattern (Figure 4.4). If bees did not regulate the ingestion of mineral diets, they would have fed randomly and displayed no clear pattern of preference, i.e. equal consumption from both diets. While this may have been the case for K, Mg, Zn, Mn and possibly Ca diets in the lower range of concentrations (Figure 4.4, 4.5, 4.6), it was not observed for the remaining minerals (Na, Fe, Cu). Bees also consistently avoided consuming high mineral diets, except in Na treatments (Figure 4.4, 4.5, 4.6). Feeding non-randomly and avoiding potentially toxic diets are behavioural responses consistent with the self-selection paradigm proposed by (Waldbauer and Friedman, 1991), in which insects benefit from selecting specific diets. In the current study, bees avoided intoxication under high mineral diets, as demonstrated by considerably low mortality rates, for example, in mineral treatments as high as 10,000 K ppm or 1,000 Fe ppm (Figure 4.11). By receiving information via gustatory sensilla on the mouthparts and postingestive feedback after mineral ingestion, bees were able to regulate high mineral intake by adjusting feeding behaviour over time.

4.6.2 *Young worker bees adjust consumption to avoid mineral intoxication*

Nectars exhibiting excessive levels of K (up to 13,000 ppm) deter honey bee foragers from floral visitation (Waller, Carpenter and Ziehl, 1972; Afik *et al.*, 2014) and feeding in artificial nectar solutions (Hagler, 1990; Afik *et al.*, 2006; Afik, Dag and Shafir, 2007). High concentrations of other minerals such as Na and Ca are known to be toxic, compromising adult bees' longevity (Herbert, 1979; Horr, 1998; A. C. Cohen, 2015). High concentrations of these minerals cause some toxicity as honey bees ingesting honeydew high in minerals were reported to experience dysentery (Imdorf *et al.*, 1985; Crailsheim and Pabst, 1988) or muscle paralysis (Horn, 1985). Young bee cohorts confined to feed on 1.0 M sucrose solutions laced with 5,000 Ca ppm over a

week also had high mortality rates (> 60 %) (Teixeira-Sousa et al. unpublished data). Here, however, I found no specific attraction to low ranges of Ca or Mg diets (Figure 4.4, 4.5). While it was evident that bees disliked both high Ca (500 ppm) and Mg (3,000 ppm) diets in the first 24 h feeding *ad libitum* (Figure 4.5), it was not clear whether they perceived these minerals in solution via gustatory sensilla.

In *Drosophila*, Ca has been recently identified as a novel mineral taste modality and different from Na as it only and exclusivelly evokes an avoidance response at high Ca levels (100 mM; 4,000 ppm Ca^{2+}) (Lee *et al.*, 2017). So far, the honey bee genome suggests a repertoire of 12 putative GRs and 21 IRs genes (Robertson and Wanner, 2006; Smith *et al.*, 2011; Sadd *et al.*, 2015), of which GRs seem to be expressed primarily in peripheral gustatory organs (Simcock *et al.*, 2017) with three GRs encoding sugars (Jung *et al.*, 2015; Takada *et al.*, 2018). Nothing has been yet confirmed for the remaining candidates nor to salt modalities. At the celullar level, electrophysiological studies attemped to examine the gustatory responses to divelent ions (e.g. Mg^{2+}, Ca^{2+}) in young adult honey bees (Whitehead and Larsen, 1976; Whitehead, 1978), blowflies (Evans and Mellon, 1962) and butterflies (Inoue *et al.*, 2012), but delivered no clear results. Still, whether similar Ca sensing pathways, which result in behavioural avoidance of high Ca diets and no specific attraction to low Ca, apply to honey bees awaits confirmation. In addition, the decrease in the total volume consumed as function of increasing concentrations (Figure 4.7) indicates that postingestive feedback mechanisms may be taking place. Ingestion of Ca and Mg diets may have triggered internal sensing mechanisms that lead to a reduction in feeding.

Similarly to grasshoppers and locusts (Bernays and Lee, 1988; Lee and Bernays, 1990; Champagne and Bernays, 1991; Trumper and Simpson, 1993, 1994; Cease *et al.*, 2016), honey bees as well demonstrate avoidance behaviours for concentrated saline solutions and other toxic substances (Ayestaran, Giurfa and De Brito Sanchez, 2010; Liu and Liu, 2010; Wright *et al.*, 2010; Hurst, Stevenson and Wright, 2014; Desmedt *et al.*, 2016). The mechanism for the reduced intake of excessive levels of salt could be mediated by hemolymph salt titers, which in turn modulate gustatory sensitivity

and subsequent feeding responses (Simpson and Raubenheimer, 1993; Trumper and Simpson, 1994). Bees are able to adjust the food passage from the crop to the midgut (Blatt and Roces, 2001) and since the midgut regulates nutrient absorption in insects, it could act as an internal nutrient sensory organ (Miyamoto, Wright and Amrein, 2013). Grasshoppers increase P excretion rates to compensate feeding on high P diets (Zhang *et al.*, 2014) and, although similar mechanisms on bees have not been reported yet, I noticed that bees under high salt diets, particullatly high K, showed increased defecation (results not shown). Further studies are necessary to determine the physiological mechanisms regulating salt intake in honey bees.

Behavioural preferences for specific salts have been examined mostly in vertebrates (Joshua and Mueller, 1979; Tordoff, 1992, 1994; Bachmanov, Beauchamp and Tordoff, 2002), which have higher requirements for Ca than insects (Allen Carson Cohen, 2015). Work performed in mice, for instance, showed that different minerals can elicit distinct taste sensitivities that may shift choices; also, it has been postulated that different minerals might be regulated by independent homeostatic mechanisms (Denton, 1982; Tordoff, 2001; Bachmanov, Beauchamp and Tordoff, 2002; Tordoff *et al.*, 2008).

Few studies have provided evidence for behavioural regulation of salt intake in insects (Trumper and Simpson, 1993, 1994; Harrison *et al.*, 2014; Cease *et al.*, 2016; Judd *et al.*, 2017). In honey bees, preference for major salts have only been examined for forager bees. Butler tested $MgCl_2$ saline solutions up to 0.92% (~9,200 ppm), though only found that foragers chose distilled water regardless (Butler, 1940). Besides, there was no attraction to Mg diets, only rejection at much lower levels (3,000 ppm). Recently, other studies reported that NaCl and $MgCl_2$ in water individually were phagostimulatory at 1.5 %, but also KCl in less extent (0–1.5 %) (Lau and Nieh, 2016). While, bees in that study were only tested for antennal responses with no feeding involved, levels as high as 1.5% in pollen or nectar would only be likely for K. Furthermore, 1.5% K in nectar has been found to deter honey bee foragers (Hagler, 1990; Afik *et al.*, 2006, 2014). Bees are more likely to accept and respond to less phagostimulatory and toxic solutions when harnessed then when

free-flying (Ayestaran, Giurfa and De Brito Sanchez, 2010; Desmedt *et al.*, 2016). In the current study, young bees also rejected high K diets (10,000 ppm; 1%), which is consitent with the previous studies. It is worth noting that the highest concentrations tested here are unlikely to be found in bee-collected pollen and even less likely in nectar/honey in unpolluted areas (with possibly the exception of K) (Nicolson and Thornburg, 2007; Nicolson, 2011; Morgano *et al.*, 2012; Filipiak *et al.*, 2017). For each mineral, I tested concentrations below, about and above the mean levels reported in pollen to gain insights on how bees respond to minerals in food over a broad range. One study, by means of behavioural and electrophysiological approaches in honey bees, proposed the existence of at least one salt cell in the tarsomere sensilla responding specifically to low KCl concentrations (0.01 and 0.1 mM; 0.39 and 3.91 K^+ ppm) (De Brito Sanchez *et al.*, 2014). In feeding tubes used in this study, if sufficient surface tension occurred, liquid diet solutions could be slightly exposed to contact with bees' taste sensilla on their tarsi, which would serve gustatory detection. However, it cannot be ascertained whether this was the case in this set up. Surprinsingly, rather than displaying attraction and preference to low and avoidance to high concentrations of K, I found no clear preference for low K diets (Figure 4.4, 4.5). K is the dominant mineral in bee-collected pollen and hymenopteran hemolymph (Natochin and Parnova, 1987; Somerville and Nicol, 2002; Morgano *et al.*, 2012) and, therefore, is nutritionally more relevant to insects compared to mammals (Cohen, 2015).

Consumption in the lower range of K diets increased with concentration, indicating a seemingly stimulation of feeding with maximum consumption reached for 1,000 K ppm (0.1% K) (Figure 4.7, Salt Diet). Consistent with this, fresh body weights of bees fed high K diets were significantly higher compared to control bees (Figure 4.10). Although no correlation analysis between mineral intake and weight gain was performed, it seems reasonable to suggest that an increase in feeding can lead to an increase in total body weight. Alternatively, this extra weight rather than being converted into tissue (unlikely for mineral nutrients), was possibly accumulated in the rectum for later excretion. Nevertheless, salt deficiency and

subsequent depression of feeding in locust nymphs reduced the conversion of food to body weight (Trumper and Simpson, 1993).

4.6.3 *Bees mostly preferred and consumed of Na diets at increasing concentrations*

Na is important for metabolic and physiological roles such as osmoregulation and tissue function (Hodgkin, 1951; Barton-Browne, 1964; Mullen and Alvarado, 1976; Nicolson, 1990; Zeiske, 1992; Emery *et al.*, 1998; Bourque, 2008). The role of dietary NaCl in preference-rejection behaviours has been subject of studies in fruit flies larvae and adults (Niewalda *et al.*, 2008; Russell *et al.*, 2011), kissing bugs (Pontes, Pereira and Barrozo, 2017), butterflies (Inoue *et al.*, 2012) and rats (Contreras and Kosten, 1983). Interestingly, in this study and at this range of concentration (5, 50, 500, 1,000 ppm) bees preferred Na diets across all concentrations. In contrast to all the remaining minerals, high Na (1,000 ppm; 43.5 mM) did not deter bees but stimulated feeding the most, indicating a strong phagostimulatory power. In the kissing bug (*Rhodniu prolixus*), feeding was optimal at 150 mM NaCl (3,450 Na^+ ppm) and gustatory responses to concentrations below and above were distinct (Pontes, Pereira and Barrozo, 2017). Bees in this study preferred moderate levels of Na (500 ppm) across the whole feeding period (Figure 4.4, 4.5, 4.7). Though, high Na was the total mean preference (Figure 4.4), bees shifted diet preference and intake over time (Figure 4.5). This shift in diet preference over 24 h (day 5 to 6) was demonstrated by a decline in the total diet consumption (Figure 4.7). It is evident that, although bees may not find high Na deterrent by taste, postingestive mechanisms assisted on regulating Na intake. In this study, I did not cover the whole range of preference-aversion thresholds. However, young bees in similar feeding contexts and regimes perceived and rejected systematically Na diets as high as 10,000 ppm Na (435 mM) (Teixeira-Sousa et al. unpublished data). These data indicate that high Na diets were beneficial for the survival of bees compared to control diets (Figure 4.11). This may relate to the fact that bees ate more in general, not only Na but energy-rich sucrose as well. Lau and Nieh, also reported that water foragers preferred solutions wih 1.5 %

NaCl (Lau and Nieh, 2016), which is above the average concentrations found in natural water sources. Na is often limiting to herbivorous insects due to low Na contents of plant tissues (Kaspari, Yanoviak and Dudley, 2008; Kaspari *et al.*, 2009). Recently, Filipiak et al. highlighted the importance of inorganic nutrient proportions in pollen to ensure a chemically balanced diet for honey bees. These authors reported limitations of S, N, P, K, Cu and Zn in some pollen types, especially of Na. By comparing elemental composition of bee-collected pollen and adult bee bodies mineral contents across castes, they estimated that Na concentration in pollen was consistently low in relation to bee body contents (workers: ~700 ppm; queens: ~1,000 ppm). This is, taken bee body composition as a proxy for nutrient requirements, then Na available in pollen may not be sufficient to match bee nutritional needs (Filipiak *et al.*, 2017). Forager bees from colonies fed pollen substitutes deficient in single essential amino acids were able to counter specific nutritional limitations by preferentially consuming complementary diets over the same or similar foods (Hendriksma and Shafir, 2016). In locust nymphs, insufficient amounts of minerals in food hampered normal development (Dadd, 1961), and induced a decrease in feeding possibly due to low palatability of food (Trumper and Simpson, 1993). In honey bees, specific salt limitations are likely to be counterbalanced by adapting foraging behaviour towards other sources, such as water (Bonoan *et al.*, 2016). These imbalances are function of season and pollen availability; 1 % Na water solutions were preferred during spring/summer, but not as much in autumn; 1 % Ca, Mg and K water solutions were favourably consumed, instead (Bonoan *et al.*, 2016).

4.6.4 *Young worker bees show limited behavioural regulation of intake at low Zn and Mn diets, but not at high concentrations*

The role of metals in bee behaviour and health have been mostly investigated within the framework of environmetnal toxicants. Honey bees and hive-derived products are regarded as bioindicators for heavy metal contamination (Van Der Steen, de Kraker and Grotenhuis, 2012; Formicki *et al.*, 2013; Herrero-Latorre *et al.*,

2017). Whereas metals such as Fe, Zn, Cu and Mn occur at physiological levels and are essential nutrients, others are xenobiotics even at trace levels (Pb, Cd, Cr, Al). The effect of metals in the feeding behaviour of honey bees is unknown. Data here demonstrated two clear preference patterns, one for high Zn and Mn avoidance, and other non-monotonic concentration-response thresholds for Fe and Cu diets (Figure 4.4).

Young bees were able to detect high Mn and avoided its consumption across the whole assessment period. However, it is not clear whether behavioural regulation is taking place at low range Mn diets because young bees in 1, 10 and 50 ppm Mn treatments did not show a preference for neither Mn nor sucrose, consuming equally from both diets (random feeding) (Figure 4.4, 4.5). Mn concentration also did not affect total diet consumption (Figure 4.8). I found no consistent reports in the literature evalutaing the specific effects of Mn ingestion in feeding responses; for a review on ingestion behaviours of metals in insects see (Mogren and Trumble, 2010). Nonetheless, Mn can occur in natural environments in high concentrations along with other metals (Boyd, 2009). Honey bees foraging on blueberry pollen containing high levels of Mn were more prone to bacterial infection (Wardell, 1982), although no specific association was demonstrated. High levels of Mn have been reported in bee heads (Nation and Robinson, 1971a); for review see (Black, 2006; Manning, 2016). Ingestion of high levels of Mn (50 mM; 2,747 ppm Mn^{+2}) has been associated with precocious onset of foraging behaviour (Søvik *et al.*, 2015). Other studies further proposed that dietary Mn modulates brain biogenic amine levels in honey bees and fruit flies via *malvolio* gene, which has been implicated in feeding-related behaviours (Ben-Shahar, Dudek and Robinson, 2004; Søvik *et al.*, 2015, 2017). To note that concentrations used it the previous study are far beyond the rejection threshold demonstrated in this current study (9.10 mM; 500 ppm Mn^{2+}).

Zn is relevant for the antioxidant metabolism as an enzyme co-factor of superoxide dismutase (SOD) and is a component of insect cuticle (A. C. Cohen, 2015; Zhang *et al.*, 2015; Marreiro *et al.*, 2017). Zn levels 30–75 $mgKg^{-1}$ added to 50 % sucrose solutions were reported to increase the activity of Cu/Zn-SOD and, by

association, antioxidant capacity in young worker bees (Zhang *et al.*, 2015). These authors delivered *ad libitum* Zn diets in concentrations within the range of those tested in the current study. Yet, they did not evalutate nor mentioned any effects of Zn on worker feeding responses. In my experiments, high Zn (500 ppm) was sufficient to deter bees. Formicki et al. assessed the mineral concentrations of several bee products in metal contaminated areas. For Zn, they found maximum levels of < 6 ppm in honey and < 150 ppm in pollen (Formicki *et al.*, 2013). Those concentrations are within the lower range of Zn diets tested here, but still below the highest level (500 ppm). Kazemi-Dinan et al. found that specialist herbivores such as the butterfly *Pieris napi* and the beetle *Phaedon cochleariae* learned to reject feeding on leaves high in Zn (> 1,000 $mgKg^{-1}$) (Kazemi-Dinan *et al.*, 2014). In the desert locust (Schistocerca *gregari*), Zn-supplemented foods (500–5,000 $mgKg^{-1}$) sharply reduced feeding as a result of postingestive learned food aversions (Behmer *et al.*, 2005). Zn reduces survival of some insects, as observed in a study of the generalist moth *Heliothis virescens* when reared in media supplemented with high Zn levels (> 1,200 $mgKg^{-1}$) (Kazemi-Dinan *et al.*, 2014). In contrast, no rejection of high Zn food was observed in the green peach aphid *Myzus persicae*. Diets amended with 1,120 Zn mgL^{-1} turned out to favour growth and reproductive traits in generalist insects (Stolpe and Muller, 2016). Also, both larvae and adult fruit flies showed peferences for low Zn (< 30 mM; 1,960 ppm Zn^{2+}) diets, whereas high Zn (70 mM; 4,577 ppm Zn^{2+}) deterred feeding (Bahadorani and Hilliker, 2009). In my work, bees did not show any specific preference for low concentrations of Zn in sucrose diets (Figure 4.4, 4.6). Furthermore, in contrast to mentioned literature, honey bees did not exhibit a strong and consistent food aversion to diets containing 500 ppm Zn. In this study, whether young bees tasted Zn in sucrose diets or whether they regulated its ingestion postingestivelly, even at high levels, could not be verified with confidence. It could be also the case that the range of concentrations tested are not biologically important to young adults. I found no negative effects of high Zn on young bee survival (Figure 4.11). Possibly, if higher concentrations were to be tested and for longer periods, a negative effect on food consumption and survival would be expectable. Although,

sample size used in these assays (N= 300 bees/concentration) was large, and the procedures systematic for all treatments, I would be keen to repeat Zn assays to confirm or not these current results.

4.6.5 Using the Bertrand's rule principle as a tool to predict optimal mineral intake through behavioural regulation

Feeding responses to Fe and Cu diets were rather interesting as, at the range tested, I found a non-monotonic dose-response preference-aversion threshold that resembles the Bertrand's rule relationship (refer to Figure 1.4, Chapter 1). Young bees increased consumption of Fe or Cu diets as function of concentration (Figure 4.4). According to this model, the increase is followed by a "plateau" that represents the optimal range of mineral intake and concentrations maintained by homeostatic mechanisms (Bertrand, 1912; Mertz, 1981). For both Fe and Cu diet treatments, the optimal preference threshold (and maximum intake) was observed at 10–100 ppm (179–1,790 μM Fe^{3+}) and 5–50 ppm (78.7–787 μM Cu^{2+}), respectivelly. Beyond these concentrations, high Fe (1,000 ppm) and high Cu (500 ppm) diets were visibly deterrent and induced a decline in consumption. Bahadorani and Hilliker investigated the feeding and oviposition behaviours of *Drosophila* flies. They found increased oviposition as function of metal concentration with flies laying eggs preferencially in media supplemented with 1 mM Fe (55.9 ppm Fe^{2+}) or 2 mM Zn (131 ppm Zn^{2+}). Beyond these optimal concentrations, the number of eggs in each media decreased. The same study showed that both larvae and adult flies preferred to feed on diets low in Fe (< 30 mM; 1,680 ppm Fe^{2+}) or Cu (1 mM; 64 ppm Cu^{2+}) while avoiding those high in Fe (40–70 mM; 2,234–2,910 ppm Fe^{2+}) or Cu (20 mM; 1,271 ppm Cu^{2+}) (Bahadorani and Hilliker, 2009). Here, bees visibly rejected diets high in Fe or Cu. I argue that bees were able to regulate dietary intake of Fe or Cu around an optimal range of concentrations by means of both gustatory and postingestive feedbacks. At low and modest levels, Fe stimulated feeding whereas 1,000 ppm Fe became distastetul and consumption declined (Figure 4.4, 4.5, 4.6). High Fe also

deterred feeding to the largest extent compared to all the other mineral treatments. Young bees ate the least volume of diet recorded (Fe 1000: 8.89 μL/bee), while increasing consumption of the paired diet – sucrose only. Water consumption within each mineral group was not expected to change at first. However, ingestion of concentrated mineral diets may promote water consumption, for example, to dilute concentrated saline solutions ingested and prevent intoxication or other detrimental effects on health. In fact, water consumption in high Fe treatments increased significantly compared to control cohorts (Figure 4.9). Additionally, these bees did not die due to starvation nor intoxication as either fresh body weights and survival did not differ from control bees (Figure 4.10, 4.11, and S13 Figure, Appendix A).

This optimisation of intake dependent on concentration implies that Fe intake must not exceed a certain threshold even for concentrations lower than those triggering rejection (100–1,000 ppm Fe^{3+}). This may well relate to the intrinsic redox activity of Fe to generate free radicals through Fenton reactions (Ray, Huang and Tsuji, 2012). Though I did not test specifically for that in this study, ingestion of increasing levels of Fe is likely to boost free circulating Fe rendering bee tissues more susceptible to oxidative stress. It has been reported that Fe-induced oxidative stress impairs olfactory learning and memory in a dose and time-dependent fashion in honey bees (Farooqui, 2008). Nevertheless, the fruit fly, and possibly bees, evolved molecular mechanisms to import, sequester and utilize iron efficiently; for review see (Locke and Nichol, 1992; Nichol, Law and Winzerling, 2002; Tang and Zhou, 2013). If this is the case, postingestive feedbacks such as those may have contributed to the regulation of Fe intake in this study and prevented higher death rates.

Similar to Fe diets, Cu consumption was optimised. Low Cu diets stimulated feeding whereas high Cu deterred bees. In spite of the fact that bees rejected feeding on high Cu diets, they still consumed ¼ of the total volume ingested per bee, which was sufficient to induce the highest mortality recorded across mineral tretaments (40 % at day 6) (Figure 4.11). Young bees were able to self-select and consume within an optimal range of Cu diets in a dose-response relationship. High mortality rates in high Cu diets beyond optimal range confirms Bertrand's rule postulating that

mineral intake decreased with rising concentrations that become toxic (Bertrand, 1912; Mertz, 1981) and, therefore, increase the risk of bees dying.

Previous research tested the toxic effects of Cu in honey bees. In laboratory-based experiments, Di et al. reported a LC50 of 6.97 mgL^{-1} Cu for bee larvae, but also that metal contamination in food affected both larvae and adults survival in a dose-dependent manner (Di *et al.*, 2016). These authors also reported that survival and motivation to feed in harnessed foragers were severely affected 24 h after being force-fed a single dose of 512 mgL^{-1} Cu in 50% sucrose solution (Di *et al.*, 2016). Hladun et al. fed exclusivelly honey bee colonies with sugar syrup and pollen patties spiked with a single concentration of several metals to evaluate its toxic effects at the colony level. The extent of the effects and colony traits affected depended on metal identity. They found that Se-treated hives (0.6–6 $mgKg^{-1}$) had reduced worker weights and Cu-treated hives (25–50 $mgKg^{-1}$) showed poor pupal survival and, subsequenlty, decreased worker populations (Hladun *et al.*, 2016).

From the current data, it can be confirmed that young bees have a innate sense of taste that renders, for example, all Na, low Fe and low Cu diets more attractive. These diets exhibited stronger phagostimulatory power and thus stimulated feeding within an optimal range of intake.

4.7 Conclusion

By screening over a range of minerals, this study documented for the first time evidence for behavioural regulation of mineral salt intake, perference-aversion thresholds and survival of young adult honey bees. Importantly, this study confirmed Bertrand's rule as a reliable framework to predict optimal mineral intake through behavioural regulation in dietary choice assays. More, that each mineral nutrient has its specific impact on feeding responses of bees that differ in the range of preferred concentrations; each mineral can also be potentially toxic if ingested over

the optimal range of concentrations within which behavioural and physiological mechanisms act best to maintain nutritional homeostasis (Mertz, 1981).

With all due limitations, this study revisited an overlooked, but relevant, area of bee nutrition and paves new ground information to support further research in this topic. Further research should be conducted to ascertain other feeding mechanisms taking place in the regulation of mineral intake in bees. The next Chapter will summarise the main motivations, accomplishments and limitations of this thesis, while casting light into future work and practical implications of studies such as this.

4.8 Acknowledgments

The execution of this work during intensive summer seasons was possible due to the much appreciated assistance provided by Robyn Darnell over one summer placement for undergraduate students in 2015. Also, I would like to thank Malcom Brian Thompson for beekeeping assistance and Mushtaq Al-Esawy for beekeeping, lab assistance, and sporadic data collection.

5

Concluding Remarks and Outlook

Chapter 5

Concluding Remarks and Outlook

5.1 Abstract

In this last Chapter (finally), I restate the motivations for conducting this work to begin with. Next, I summarise the main findings obtained experimentally and pinpoint its due limitations (no research work is flawless). Lastly, I contextualize this novel piece of information in the framework of insect behavioural regulation towards nutritional homeostasis. Here, we shall discuss what I did not know before, what we know better now, either supported or not supported by current data, what we still do not know (a lot), but most importantly, which implications or opportunities this study conveys. This work was rationalised with two main goals. The first was to revisit honey bee mineral nutrition, a neglected topic, and to develop methods for studying it. The second, and most important, was to ascertain whether adult worker bees (central for colony nutrition) employ mechanisms to regulate the intake of mineral salts. These micronutrients are prevalent in pollen and essential for somatic maintenance. Untill recently, information was taken for granted, tended to be vague, scattered and outdated. In Chapter 3, I assessed the gustatory behaviour to determine whether foragers detect and how they respond to salts and metals in solution (appetitive/aversion); next in Chapter 4, I studied whether young workers detect and select appetitive or reject concentrated mineral diets. This feeding behaviours underlie optimisation of intake and, therefore, is indicative of behavioural regultation. Altogether, I am confident I have succeeded in shining a spotlight on the importance of mineral nutrients in bee diet and the biological

significance they exert on adult workers' preingestive pathways associated with nutritional regulation.

5.2 Motivation and Main Findings of this Study

This study was motivated to determine the extent of mineral salt perception, feeding preferences and intake regulation by adult worker bees. In laboratory-based assays, I then assessed the behavioural responses associated with feeding behaviour of young worker honey bees to the eight most prevalent minerals in pollen (salts: K, Na, Mg, Ca; metals: Fe, Zn, Cu, Mn).

In Chapter 3, using the classical PER approach, I tested the gustatory responses (antennae/proboscis) of forager bees to single minerals in either water or nectar-like solution (1.0 M sucrose). I found that foragers (mixed-age) can detect individual salts/metals mineral salts, though responses depended on mineral identity. Overall and as expected, bees found low mineral levels in water phagostimulatory. But when in sucrose solutions, only high Mg, Fe and Cu were clearly detected and rejected. When stimulated on the mouthparts, in contrast to the antenna, bees were less responsive (more sensitive) to minerals in water/sucrose, especially at high concentrations.

In Chapter 4, in the context of cohorts of bees given a choice between two diets, I assessed the feeding preferences, responses (consumption) and survival of newly-emerged bees to individual mineral diets over their first six days of adulthood.

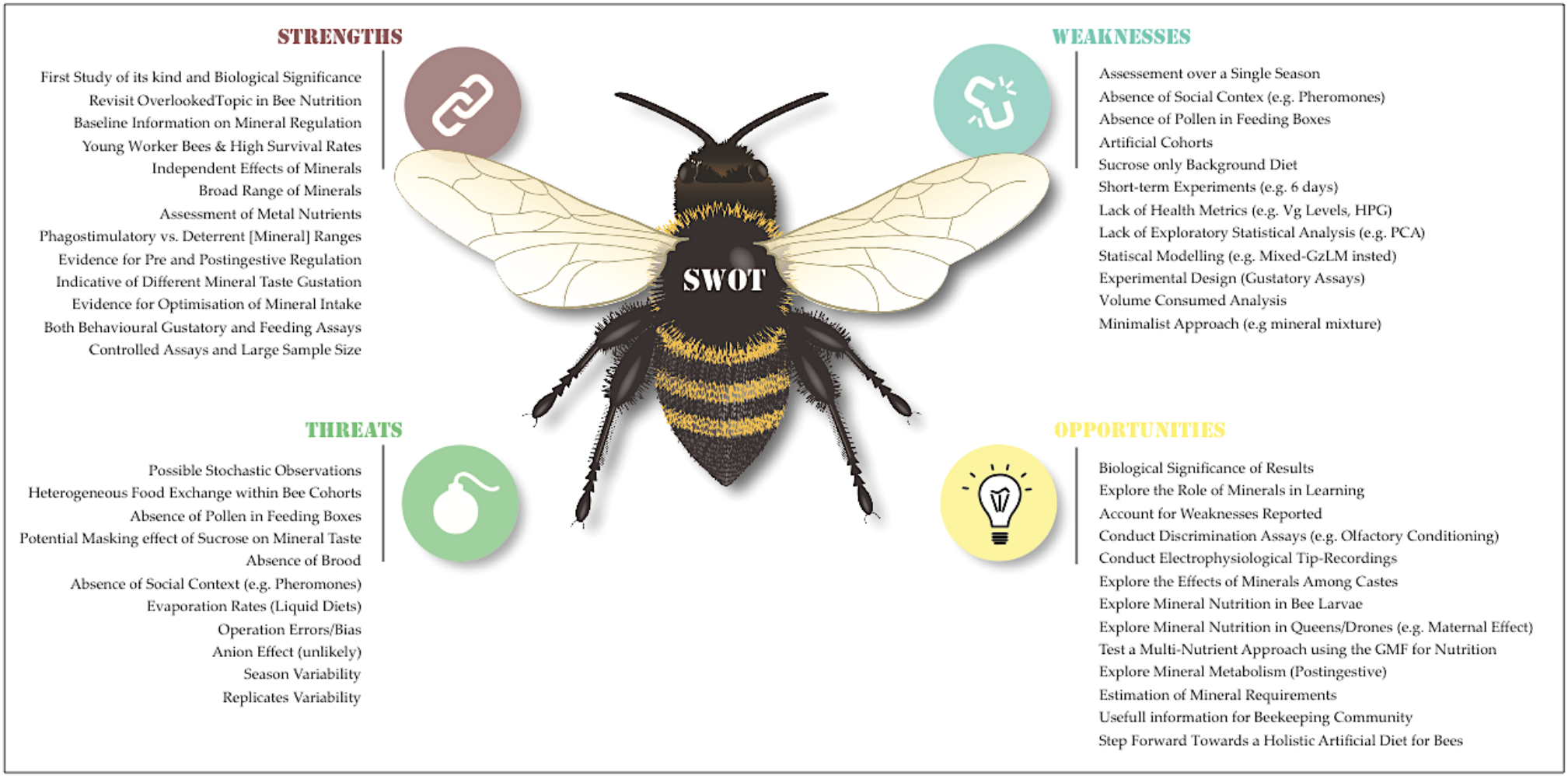


Figure 5.1 Diagram depicting the SWOT analysis of the current research project on mineral gustation and behavioural regulation of intake in adult worker bees under laboratory settings. (SWOT: Strengths, Weaknesses, Opportunities, Threats). These notes represent the overall conclusions after performing gustatory assays using PER and drinking assays in Chapter 3, and two-choice feeding assays in Chapter 4. Abbreviations: Vg, vitellogenin; HPG, hypopharyngeal glands; PCA, principal component analysis; GzLM, generalized linear models; PER, proboscis extension reflex; CS, conditioned stimulus; US, unconditioned stimulus; GMF, geometric framework for nutrition. @ Credits to Almudena Clemente for the bee artwork.

By delivering a choice between sucrose alone vs. sucrose laced with a single mineral at four levels of concentration (broad range: 0–10,000 ppm), I tested whether bees preferred a "salty" vs. "unsalty" solution; how dietary minerals and concentration influenced consumption and survival of bees. I was able to verify whether young workers demonstrate regulation of mineral salt ingestion through behavioural adjustments in consumption: 1) young bees not tending larvae nor the queen perceive and select specific minerals in food; 2) not all minerals act as phagostimulants at low levels but act as deterrents at sufficiently high levels; 3) young bees show behavioural regulation of mineral intake, but not all are regulated to the same extent; and 4) different minerals evoke different gustatory responses.

This work is the first to evaluate gustatory responses and the dietary self-selection of metal nutrients, and the second to assess salt preferences of adult worker honey bees in a choice context. In Chapter 1, I provided an overview of the main concepts understudied, the rationale of this work and the structure of this thesis. Then, Chapter 2 addressed a series of pilot experiments necessary to optimise data collection and data processing from feeding assays described in Chapter 4. Those preliminary assays, helped to confirm a suitable range of concentrations for each mineral that supported both feeding and survival in appropriate feeding boxes (acrylic) (Study 1). Also, I found the relevance of shuffling boxes across shelves to avoid biased evaporation of diets (Study 2), but also that diet tubes' position within the feeding box affected the magnitude of consumption measurements (Study 4). I tested different methods to account for evaporation loss from diet solutions (Study 3). To produce more reliable consumption measurements, cut-off thresholds were established for the number of bees per feeding box (Study 5). Chapters 3 and 4 covered the main set of experiments that tested the behavioural gustatory responses to minerals in solution, and assessed behavioural regulation of mineral salt intake, respectivelly. This present Chapter, summarises the work documented throghout and emphasises the biological significance of this study for bees and its practical implications.

The current study lays the groundwork for exploring mineral salt nutritional requirements, feeding preferences and regulatory mechanism of regulation of salt intake.

5.3 Limitations of this Study

For as much as the design and execution of the experiment in this work were taken as carefully as possible, due to my eagerness to start working back in 2014 and time constraints over the course of the project, this study displays some limitations. First, I believe now that the experimental desing for the gustatory assays in Chapter 3 could have been better cared for. As Lau and Nieh pointed out (Lau and Nieh, 2016), high salt can be an anti-reward, but low salt is appetitve and contrasts to similar approaches in which high sugar is good, but low sugar concentrations are not as attractive. With the knowledge and insights I had then, when I first designed these gustatory assays, the stimuli presented to the bees was executed in a concentration gradient from low to high salt in accordance to sucrose responsiveness assays (Scheiner, Page and Erber, 2004). To some extent, this strategy may have accounted for the observed ceiling and floor effects; refer to results in Chapter 3. Similarly, the internal state of forager bees may have influenced gustatory responses as some treatments were conducted later in the season.

In the feeding assays documented in Chapter 4, some limitations can also be underlined. For example, feeding cohorts and diets were minimalist with regards to the composition of diets (sucrose based), pollen patties that assist young bees to grow in their first days of adulthood (Crailsheim, Schneider, Hrassnigg, Brosch, *et al.*, 1992) were absent, and consumption was reported as volume only. Moreover, young bees were deprived of the social context, but my estimatios are that the behavioural responses reported here would follow a similar profile but sharper. Nonetheless, this remains to be investigated in more contextualized environment. Also, it is good practice to assess other health parameters beyond survival rates such as the composition of the hemolymph, the levels of vitellogenin (Vg), the growth of the

HPG (though pollen was not provided to these cohorts), and the mineral body composition of bee body parts are some examples. These parameters have been used in similar research approaches (Judd and Fasnacht, 2007; Huang *et al.*, 2014; Stabler *et al.*, 2015; Judd *et al.*, 2017; Traynor *et al.*, 2017; Corby-Harris *et al.*, 2018). If all or some of these parameters would have been included in this study design, a more comprehensive understanding of the effects of mineral nutrition and regulation in adult workers would certainly be possible. However, this would only be possible (for a similar time-window) if the experiments were narrowed down to a single or a couple of minerals investigated. Besides, larger beekeeping facilities and human taskforce would be a prerequisite. Nevertheless, I chose to conduct a screen analysis and to cover a broader range of minerals (the most prevalent in bee-collected pollen) and, thus, with greater biological importance. One other possible limitation extends to the statistical approach used. For example, nor in the pilot assays nor after, I conducted a principal component analysis (PCA) on my data, which is a common and recommended pratice in data exploration. I learned about this methodology when I was collecting data for the scaled up experiments. The main decisions were already established. Therefore, these limitations are now advanced as recommendations for the prospective work.

5.4 Gap in the Literature. What we knew so far

Identifying and selecting the correct food (quality and quantity) is critical to optimal fitness. This is accomplished through behavioural (taste and feeding) and postingestive feedbacks when an insect detects and selects foods (Chapman and de Boer, 1995; Simpson *et al.*, 2004). The social existence of honey bees results in a partitioning of specific nutritional needs. As such, adult workers are central in securing colony nutrition. Food selection is accomplished twice in bees: foragers first select and gather nectar/pollen back to the hive; then, young workers are the ones actively consuming nectar and pollen necessary to produce glandular secretions aimed to feed larvae and the queen (a form of lactation in insects) (Crailsheim,

Schneider, Hrassnigg, Bühlmann, *et al.*, 1992; Lass and Crailsheim, 1996; Toth and Robinson, 2005; Wang *et al.*, 2014). Adult workers can regulate protein and lipid intake (Altaye *et al.*, 2010; Pirk *et al.*, 2010; Paoli *et al.*, 2014; Stabler *et al.*, 2015; Vaudo *et al.*, 2016, 2017), and adjust their feeding behaviour to control for nutrient deficiencies at the colony level (Zarchin *et al.*, 2017).

Mineral salts are important micronutrients, which are often limiting to phytophagous insects and other herbivores as most subsist off low Na diets typical of plant tissues (Kaspari *et al.*, 2009; Dudley, Kaspari and Yanoviak, 2012), and bees included (Filipiak *et al.*, 2017). In the context of Na deficiency, a wealth of species engage in a motivated behavioural state that drives them to seek and ingest foods that contain Na; for a review refer to (Schulkin, 1991; Hurley and Johnson, 2015). This behaviour is an innate regulatory mechanism that ultimately directs animals to seek, detect and ingest specific foods to restore Na levels. In insects, salt-seeking behaviours have been reported in ants (Kaspari, Yanoviak and Dudley, 2008; Dudley, Kaspari and Yanoviak, 2012; Hernández *et al.*, 2012), locusts (Shen *et al.*, 2009), solitary bees (Bänziger *et al.*, 2009; Abrol *et al.*, 2012) and social bees (Butler, 1940; Bonoan *et al.*, 2016; Dorian and Bonoan, 2016; Bonoan, O'Connor and Starks, 2018). Behaviours such as these, often termed as puddling, are thought to be a form of supplementary feeding targeted at specific micronutrients; for a review refer to (Molleman, 2010). Yet, puddling is most often described in some species of moths and butterflies, especially males (Adler and Pearson, 1982; Smedley and Eisner, 1996). Puddling for salt in lepidopterans stems from the fact that is thought to increase female reproductive success via paternal contribution of Na to eggs (e.g. nuptial gifts) (Adler and Pearson, 1982; Pivnick and McNeil, 1987; Smedley and Eisner, 1996; Molleman *et al.*, 2005). In male pipevine swallowtail butterflies (*B. philenor*), Na consumption has been suggested to increase male neuromuscular activity and, therefore, supporting a more vigorous courtship and mating success (Mitra *et al.*, 2016). In my understanding, salt intake observed in certain male lepidopterans could be an indirect mechanism that prevent future generations from

salt deficiency, and not an immediate regulation of salt intake. It is unknown whether this occurs and affects reproductive success of queens in honey bees.

Salt and metal nutrients are likely to be important for worker caste individuals in a social insect colony, as their food collection behaviour impacts the performance of the whole colony. Yet several aspects of mineral nutrition and feeding have rarely been addressed, especially in social insects. Long-lasting works established that minerals were only required during development in minute amounts in pollen, and not necessary for adult bees (Nation and Robinson, 1968; Haydak, 1970; Herbert and Shimanuki, 1978; Brodschneider and Crailsheim, 2010). However, salts are recognized to elicit specific gustatory and feeding responses in other animals. Often, low salt stimulate feeding and high salt inhibits feeding (Dethier, 1977; Bahadorani and Hilliker, 2009; Zhang, Ni and Montell, 2013; Pontes, Pereira and Barrozo, 2017). Beekeepers keeping the hives near the sea have witnessed honey bees foraging and collecting seawater (up to 3.5% salt, mostly Na), although observations such as these haven´t been formally described and are based on personal communications within the beekeeping community.

Not until recently, two studies reported specific preferences for major salts in water either under laboratory settings (Lau and Nieh, 2016) or semi-field framework (Bonoan *et al.*, 2016). Also, in bees, high K and P in floral nectar seem to deter flower visitation and nectar consumption (Waller, Carpenter and Ziehl, 1972; Hagler, 1990; Afik, Dag and Shafir, 2006; Afik *et al.*, 2014). On the contrary, minerals in pollen (main source of non-carbohydrates) seem to be phagostimulatory and increase consumption (Schmidt *et al.*, 1995). Whether this is specific to pollen ash contents (mix of minerals and other impurities) or to specific proportions between different minerals in pollen is not clear. In fact, two seminal works established that brood rearing is increased by the addition of pollen ash at optimal levels, i.e. mostly minerals, to synthetic diets (Nation and Robinson, 1968; Herbert and Shimanuki, 1978). Brood rearing was best between 0.5–1% pollen ash, but decreased for levels > 3% and hampered at 8% pollen ash while inducing higher worker mortality (Herbert and Shimanuki, 1978). This supports the previous assumption that minerals are

necessary for larvae development. Altogether, these studies imply that worker bees must regulate the ingestion of diets based on mineral contents that supports both brood rearing and adult survival. These reports align with others in relation to the mineral composition of royal jelly. The mineral composition of royal jelly has been reported in the range between 0.8% and 3% (Sabatini et al., 2009). In fact, levels of Zn, Cu and Fe do not seem to vary largely compared to honey and pollen, which implies that homeostatic mechanisms operate possibly at the level of hypopharyngeal glands to buffer mineral variation on honey and pollen percursors (Stocker et al., 2005; Wang et al., 2016; Balkanska, Mladenova and Karadjova, 2017).

Overall, these studies indicate that an optimal range of mineral contents either in royal jelly and pollen are necessary for adequate bee nutrition and that worker bees are pivotal on mediating that process. As such, worker bees are expected to regulate food consumption in a way that meets their own needs and the requirements for the production of jelly. Until now, whether worker bees optimised the intake of minerals from food has never been formally studied. Other authors have rather focused on the effects of metals (e.g. Se, Mn, Cu) on foraging behaviour, learning ability and mortality of individual forager bees (Ben-Shahar, Dudek and Robinson, 2004; Hladun *et al.*, 2012, 2013; Søvik *et al.*, 2015; Di *et al.*, 2016) or at the colony level (Hladun *et al.*, 2016). To my knowledge, the ingestion behaviour and gustatory responses to salts and, especially, to metals at nutritionally significant levels in honey bees has never been addressed until now.

Bees can detect major salts in water and nectar, but can they perceive metal nutrients as well? Is the perception of minerals all the same? Can bees regulate salt/metal intake to balance deficits and prevent toxicities? Perhaps minerals in pollen could be considered a reward at certain extents owing to the fact that these components are likely to be assessed directly upon contact (not requiring digestion) and, thus, stimulate feeding. Nevertheless, whether adult workers detect pollen nutrients via gustatory pathways and preingestive regulation is still controversial and warrants attention (Pernal and Currie, 2002; Cook *et al.*, 2003; Leonhardt and Blüthgen, 2012; Corby-Harris *et al.*, 2018); for a recent review see (Nicholls and

Hempel de Ibarra, 2016). Two recent studies evaluated pollen preferences of nurse bees either by the nutritional value (protein to lipids ratio) of pollen (Corby-Harris *et al.*, 2018) or by the "shelf life" (fresh vs. stored beebread) of beebread (Carroll *et al.*, 2017). In Corby-Harris et al. study, they measured the growth of the HPG and protein to lipids ratio as metrics for the nutritional value across series of pollen/diets. They found that nurse bees do not always prefer (and consume) the "most nutritious" pollen/diets (Corby-Harris *et al.*, 2018). In the second study, Carroll et al. observed that young workers would consumed fresh beebread preferentially in the first few days, otherwise accumulating stored older pollen in excess; freshly stored pollen did not endow bees with any development benefit compared to older stored-pollen (Carroll *et al.*, 2017). None of these two works ever mentioned words such as salts, minerals, ash, vitamins nor micronutrients. From my point of view, in the first study, preference reported for the "less nutritious pollen" could well relate to the content of other nutrients such as minerals, which could render pollen/diet more attractive regardless of other nutrients, which they did not test for; as for the second study, preference for the freshly-stored beebread could relate to vitamin contents. Vitamins can increase food palatability, but are thermolabile and deteriorate over time (Black, 2006; Campos *et al.*, 2008). Simultaneously, could also be the case that older stored pollen builds up in trace elements and other impurities, decreasing pollen acceptability.

The ability to regulate the intake of minerals is necessary as pollen composition varies largely across species (Filipiak *et al.*, 2017) and tends to change with season (Bonoan *et al.*, 2016; Bonoan, O'Connor and Starks, 2018), and colony demands are constant. Forager bees from colonies fed pollen substitutes deficient in single essential amino acids were able to counter specific nutritional limitations by preferentially consuming complementary diets over the same or similar foods (Hendriksma and Shafir, 2016). This suggests that, mineral imbalances in food are likely to be regulated. It is not likely that honey bees ever face such dramatic salt deprivation contexts. In honey bees, specific salt limitations are likely to be

counterbalanced by adapting foraging behaviour towards other sources, such as water (Bonoan *et al.*, 2016).

In the context of artificial feeding cohorts, the present work adds the information that nurse-like bees not tending for brood nor the queen seem able to regulate individual salts/metals in the diet. Furthermore, they have a "salty tooth" by which they were attracted to increasing concentrations of NaCl at the expense of sucrose alone, but also avoided high concentrations of most minerals. Interestingly, these data confirm that not all minerals taste the same nor are regulated in the same extent. In rats, mineral deprivation differently affected mineral salt intake, but not for all at the same extent (Tordoff, 1992). This implies that despite its differences in gustatory perception, some minerals may be co-regulated by the same mechanisms. This was not assessed here, but whether or not it would apply to bees requires further studies. From gustatory data and feeding preferences Fe and Cu produced the most conspicuous responses across contexts. Bees regulated the intake of Fe or Cu diets around an optimal concentration, suggesting that, for example, Fe and Cu are not only required but should be harmful if ingested in excess. The Bertrand's rule is, therefore, a proven resource to predict optimal intake of mineral salts through regulation of mineral diet intake (refer to Chapter 1 and 4). This model stems from the dose-response curve for essential mineral nutrients: at low doses, increased intake associates with health benefits until an optimal intake is reached; further intake at higher doses results in health costs (Bertrand, 1912; Mertz, 1981). For all data gathered here, I predict that optimal intake thresholds for young worker bees are as follow, Na: 500–1,000 ppm, K: 1000–10,000 ppm, Ca: < 50 ppm; Mg: < 30 ppm; Fe: 10-100 ppm, Cu ≈5 ppm, Zn (inconclusive), but < 500 ppm, and Mn: < 50 ppm.

Seminal studies in *Locust migratoria* nymphs have already demonstrated that salt intake can be optimised by shifts in feeding activity (Trumper and Simpson, 1993, 1994). For example, if locusts are allowed to choose between different diets varying in salt concentration, they would regulate the ingestion of a salt mixture (e.g. Wesson's Salt Mix) around a preferred concentration independently of macronutrient intake. This is no longer true if locusts are restricted to a single

concentration of salt, in which situation macronutrient intake is rather controlled, regardless how much salt they eat. If animals are confined to a single concentration on a specific nutrient they are not able to self-regulate its intake, thus, over time, they may suffer consequences of under or over ingesting that food. In fact, the costs of ingesting excesses are reduced compared to those arisen from deficiencies of one or more nutrients (Trumper and Simpson, 1993; Simpson *et al.*, 2004). A combination of responses seem to encompass dietary salt balance in locusts: shift in locomotory behaviour; innate taste response; non-associative (neophilia or habituation) and associative learning (positive or negative) (Simpson, Chyb and Simpson, 1990; Trumper and Simpson, 1993, 1994).

A powerful tool to define and explore nutritional regulation is "The Geometric Framework for Nutrition" (GMF) (Simpson and Raubenheimer, 2011, 2012). This is a modelling approach that explores how an animal solves the quest of matching their nutritional requirements in a multidimensional and variable environment, and it has demonstrated to be a reliable tool to evaluate the feeding behaviour and to quantify the nutritional requirements of animals, including humans (Simpson and Raubenheimer, 2011, 2012). If in the natural environment there is a combination of relevant nutrients that is supposed to be optimal (nutritional target, NT), animals have evolved physiological and behavioural strategies that allow them to reach nutritional optima and respect their nutritional demands at different life stages. Two other targets are envisaged in this framework: the intake target (IT), which consists of the amount of nutrients that is required to be ingested so that the animals achieve their nutritional target, and the growth target (GT), which is the proportion of ingested nutrients that will translate into growth and tissue storage. The IT will always be greater that the NT as not all nutrients are equally absorbed and the GT is estimated by subtracting the metabolic requirements to the NT (Simpson and Raubenheimer, 2012). GMF designs have allowed to explore optimal nutritional landscapes and regulatory mechanisms of several animals, among them insects, birds and mammals, with diverse feeding habits (herbivores, omnivores, carnivores). The first aim of a nutritional study is to quantify nutrient requirements of an animal at a

certain life stage. The main classical experimental approach is to estimate food intake by means of feeding assays using GMF for nutrition model designs. Feeding experiments are generally designed in two ways: choice (normally two foods are presented) and no choice assays (single diet restriction). Choice assays allow the animal to self-select and test whether it regulates nutrient intake, and if so, to what extent. This method also enables the quantification of food ingestion by dynamic monitoring (e.g. every 24 h over 7 days), enables an animal to reach its nutritional optima and IT, but does not account for the food excreted. In contrast, no choice assays confine an animal (or group of animals) to one single food that varies the ratio/concentration of nutrients, and allows estimations on how the animal regulates nutrient intake and the physiological outputs of feeding on imbalanced diets.

There is another frameworks for exploring and understanding the interaction between animals, nutrition and their environments, the Ecological Stoichiometry Framework (ES) (Elser, 2006). This framework focus on the balance of energy and multiple chemical elements in animals, specifically what is the biological significance in the elemental composition of animal bodies; how it influences growth via production of ribossomal RNA ("growth rate hypothesis") by which phosphorous (P) is pivotal; see review (Elser, 2006). As such, the GMF and the ES are the two current approaches used to disentangle the role of nutrients in shaping animals' history within their surrounding environments. So far, the GMF approach has focused mostly on the behaviour of terrestrial insects using controlled feeding assays designed for macronutrient intakes, see (Simpson and Raubenheimer, 2012). In contrast, ES approach has been focused on the distribution of key elements across different trophic levels in aquatic organisms and, as such, ratios between C:N:P are the pivotal nutrients investigated (Elser, 2006). A review exploring differencies and parallels between these two frameworks has been published recently (Sperfeld *et al.*, 2017).

Studies evaluating the behavioural regulation of mineral intake in insects using the GMF are scarce, but the existing few demonstrate the diversity of responses depending on the animal context and mineral identity. For example, termites

(Blattodea: *Reticulitermes flavipe*), a social insects with different colony structure compared to bees, has been recently reported to balance the intake of mineral nutrients by adjusting consumption rates between two complimentary foods of KCl, $MgSO_4$, and $FePO_4$ (Judd *et al.*, 2017). These insects consistently overconsumed KCl or $FePO_4$ to prevent consuming too much of $MgSO_4$. Also, two studies showed opposite responses regarding P (phosphorous) intake. In the field cricket *Gryllus veletis*, consumption among P diets was seamingly random and no positive effect on fitness arrived from ingesting P, implying that P intake is not regulated (Harrison et al., 2014). Whereas, the grasshopper *Schistocerca americana* was able to select among diets to reach an optimum intake of P that attained the best growth rate and survival (Cease et al., 2016). These hoppers, when confined to diets high in P, increased excretion rates, suggesting the existence of homeostatic mechanisms that facilitate the regulation of internal P after ingestion (Cease et al., 2016).

5.5 Prospective Work and Opportunities

An essential endeavour will be to execute a multi-nutrient approach within the GMF framework. This will enable to explore the role of dietary mineral mixtures or individual minerals relative to the dietary macronutrients (e.g. carbohydrates, lipids, proteins) and how variations in food composition or nutritional status (hunger vs. satiety), for example, influence feeding decisions and physiological mechanisms (e.g. excretion). Also, behavioural and taste sensilla extracellular recordings could be performed on the mouthparts and tarsi specifically to tackle the microstructure of mineral feeding and the neuronal gustatory responses to dietary minerals.

Further studies are needed to evaluate the impact of mineral salt mixtures on adult bees feeding behaviour; to explore how foraging specialization (water, nectar and pollen) influences mineral salt detection; to determine the impacts of mineral salt feeding on adult bee performance and brood rearing. Moreover, other studies could focus on assessing the impact of low and high mineral salt feeding during honey bee larvae development, and to assess the reproductive output of queen bees raised in

sodium/iron-enriched media, for example. Olfactory conditioning methodologies could also be employed to understand the effect of iron in bee learning (Menzel and Muller, 1996). Iron shortages in mammals, for instance, impair learning and memory (Fretham, Carlson and Georgieff, 2011), but may also affect food intake (Gao *et al.*, 2015). To disentangle whether individual minerals are in fact perceived by means of taste, discrimination assays should also be conducted using as well olfactory conditioning approaches, for example.This technique has been used in insects to test different compounds in a multitude of designs (Niewalda *et al.*, 2008; Wright, Choudhary and Bentley, 2009; Wright *et al.*, 2010; Russell *et al.*, 2011; Nicholls and Hempel de Ibarra, 2013, 2014; Simcock, Gray and Wright, 2014; De Brito Sanchez *et al.*, 2015; Muth, Papaj and Leonard, 2016; Guiraud *et al.*, 2018).

5.6 Significance of this Study and Contributions

Mineral imbalances can affect animal welfare and productivity in the livestock industries (Hidiroglou, 1982). Malnutrition, possibly assisted by mineral imbalances in bee food, may as well induce nutritional stresses and limit health and performance of honey bee stocks (Somerville and Nicol, 2002; Goulson et al., 2015). Nutritional regulation in a social colony is more complex. To maintain nutritonal homeostasis, honey bees need to do more than just become satiated, they must be able to identify and choose among pollen types, which organoleptic properties are likely to influence feeding behaviour at several levels. Despite the first insights provided by the present study, further evidence is necessary to confirm whether bees taste and discriminate specific salts/metals in pollen by means of preingestive pathways; see review (Nicholls and Hempel de Ibarra, 2016). At the cellular level, a metal-responsive transcription factor (MTF-1) has been implicated in metal perception in insects, but whether it acts at the peryphery and associates with behavioural responses is unknown (Günther, Lindert and Schaffner, 2012). Chemoreceptors at the periphery responding specifically to salts or metals in bees are unknown. Nevertheless, the work described here along with others works imply that some insects perceive

mineral composition of food and adjust their behaviour to select preferred foods and avoid less edible others. This may depend on the salt or metal identity, concentration and possibly food complexity.

5.6.1 Beekeeping Practices and Feed Supplements

By having long-lived colonies, honey bees need to collect nectar and pollen from a diversity of flowers during spring and summer season for their own nutrition, but also to provide the colony with enough food supplies overwinter (Westerkamp, 1991). Flower gaps over the year or dearth periods may cause the absence or a decrease in the nectar flow and pollen shortages. Also, during winter time, the main cause of colony deaths is not cold temperatures, but rather starvation (Somerville, 2005). As a matter of aiding colonies to thrive, and preventing colonies starvation, beekeepers provide their bees with feed supplements or substitutes (pollen, sugar or both). Other reasons for feed supplementation can be as such to increase brood rearing activity; to warrant nutritional state of the colony by ensuring pollen and nectar (sugar) income and sustain food reserves; strengthen colonies for packaging production; increase bee population for pollination services, build up colony population for spring/autumn; queen rearing; overcome crop pesticide damage and assist colonies to overcome disease, reviewed in (Graham, 2010). Feral honey bee colonies may survive and thrive unaided by man, but there should be also colony losses (~25%) (Seeley, 2010). Over the years, researchers and beekeepers tried to develop new food formulations to supplement managed honey bee colonies with proper sugar ratios and pollen/protein requirements, the telling point is that most of them are enriched by the addition of natural pollen. Formulations for pollen substitutes are based on other protein sources such as soy, wheat and yeast extracts. Mineral/vitamin supplementation have also been developed and used over the years in (Graham, 2010), but less frequently. Pollen (main source of minerals) supplements seem to be effective in maintaining brood rearing, but not necessarily stimulating it (Somerville, 2005). Several bee food supplements have been formulated upon bee

pollen and honey composition, and marketed over the years amid sugar fondants, protein mixtures, vitamin and antioxidant supplements. Most manufacturers make positive claims about these products based on absent or scarce scientific evidence. Furthermore, few may disclose information about the composition of their. In a practical approach, the effects of certain minerals such as Li (lithium) and Cu (copper) have been investigated in the context of anti-parasite properties (Bounias, Navonenectoux and Popeskovic, 1995; Wardell, Degrandi-hoffman and Hayden, 2008; Ziegelmann *et al.*, 2018). However, these studies focused only on adult bee survival and toxicological effects against the varroa mite. In these studies, despite lithium and copper salts were delivered through feeding, honey bee feeding responses and ingestion behaviours were not assessed, at least not reported.

5.6.2 Pollinators Decline and Bee Stocks

Since (Oldroyd, 2007), more studies reported about insect pollinators virtual population declines (VanEngelsdorp *et al.*, 2009; Potts, Roberts, *et al.*, 2010; vanEngelsdorp and Meixner, 2010), raising concerns about a potential supply-demand mismatch (Aizen and Harder, 2009; Potts, Biesmeijer, et al., 2010; Breeze et al., 2014). This indicates that, even though bee stocks have been increasing since the 90s, may not be sufficient to supply estimated demands for pollinator-dependent crops production (Aizen and Harder, 2009; Potts, Biesmeijer, *et al.*, 2010; Breeze *et al.*, 2014; Potts *et al.*, 2016). This circumstances pose particular concerns to the US, because *Apis mellifera* is the only honey bee species in North America (Calderone, 2012). It is, thus, not surprising why managed honey bees can contribute greatly to the world economy: 1) honey bee stocks are an established commodity (Calderone, 2012); 2) colonies can be easily kept and handled (Calderone, 2012); 3) beekeeping practices are well-developed (Atkins, Grout and Dadant & Sons, 1975); 4) each long-lived colony produces several thousands of bees (Wilson, 1971; Tautz, 2008); 5) honey bee colonies show extended foraging ranges (Hagler et al., 2011; Couvillon et al., 2015); and 6) honey bee colonies display flower constancy and are polyphagous

insects (Chittka, Thomson and Waser, 1999). The state of global pollinators, the risks they face, and the impact of pollinators systematic decline in our food security have gain much attention in these last few years. In one of the latest reviews, Potts at al. addressed five critical drivers for insect pollinators decline: 1) pollinator management and pathogens; 2) land-use and intense agriculture; 3) climate change; 4) pesticides and genetically-modified organisms; and 5) invasive species) (Potts *et al.*, 2016). One route to protect insect pollinators such as the honey bees is understanding more about them and their surrounding environment. This is one of the main drives of bee research.

To conclude, by bringing this collective piece of information aligned with previous literature and, now, with a better understanding of what is required or it is not, one realises that he/she did not solve nor ticked off any task from the bucket list, but rather contemplates another or several other avenues in need to be further explored.

Appendix A

Supplementary Information

Appendix A

Supplementary Information: Tables and Figures

In this Appendix A, I provide further Supplemental Information (SI) such as Figures and Tables that can be useful for the reader. While the main Figures and Tables are numbered sequentilally as they appear in the main text within each Chapter (Chapter 1, Figure 1.1, Table 1.2; Chapter 5, Figure 5.3, Table 5.1, etc.), Supplementary Figures and Tables are numbered sequentilally as they appear in the main text and across Chapters (Chapter 1, S1 Figure, S2 Table; Chapter 5, S12 Figure, S13 Table, etc). One Figure or Table is presented per page and sequentially as they appear in this thesis.

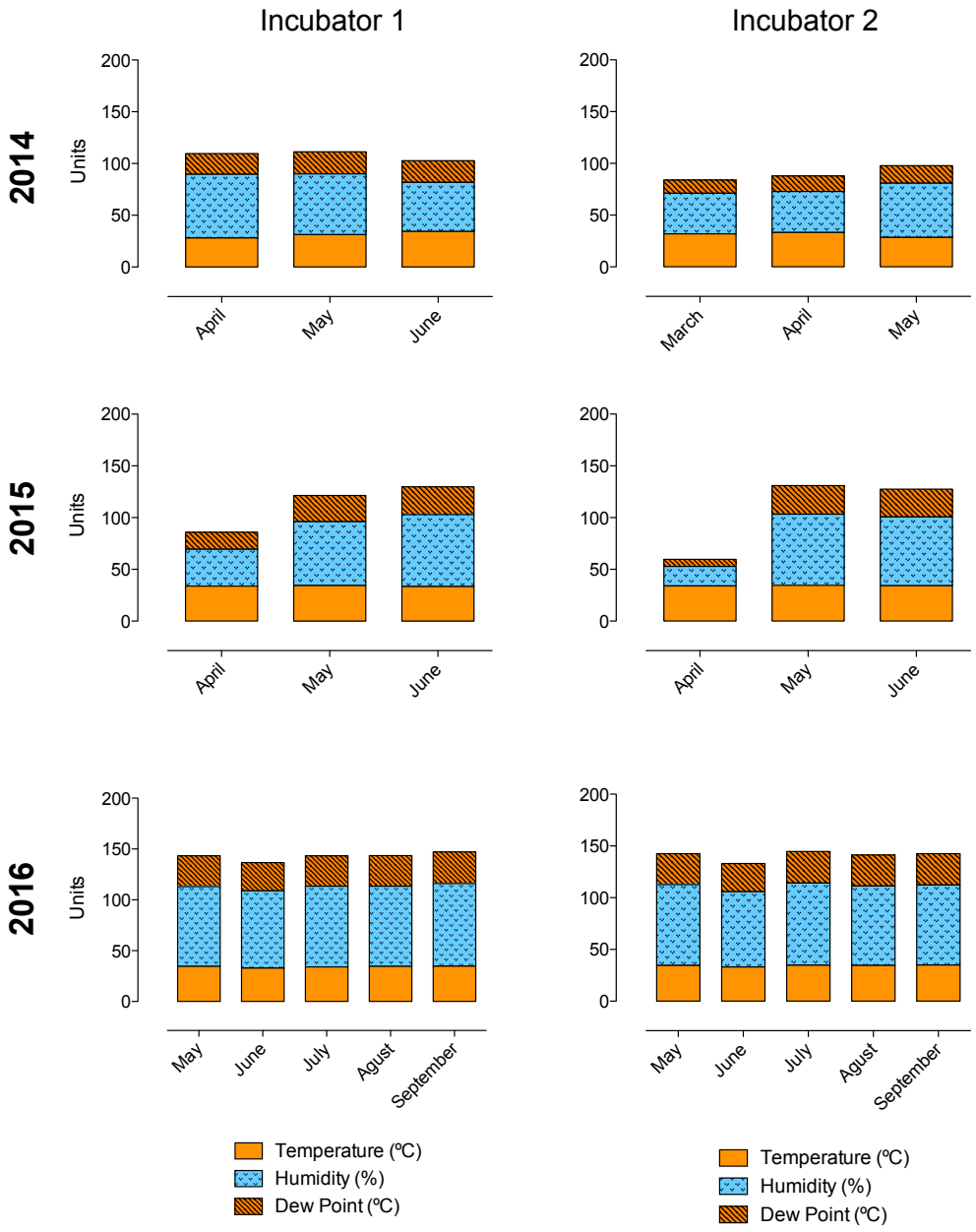
S1 Table. List of Reagents used in Behavioural Experiments to prepare Diet Solutions (refer to Chapters 2, 3 and 4).

Formula	Reagent name	Reference	CAS No.	MW*
KCl	Potassium Chloride	Fisher Scientific P/4240/60	7447-40-7	74.56
NaCl	Sodium Chloride	Fisher Scientific S/3160/60	7647-14-5	58.44
$MgCl_2.6H_2O$	Magnesium Chloride Hexahydrate	Sigma M2393	7791-18-6	203.3
$CaCl_2.2H_2O$	Calcium Chloride Dihydrate	Fisher Scientific C/1500/53	10035-04-8	147.0
$CuO_4S.5H_2O$	Copper (II) Sulfate Pentahydrate	Sigma C8027	7758-99-8	249.7
$CuCl_2.2H_2O$	Copper (II) Chloride Dehydrate	Sigma C3279	10125-13-0	170.5
$ZnCl_2$	Zinc Chloride	Sigma Aldrich 229997	7646-85-7	136.3
$C_6H_8O_7 \cdot xFe^{3+} \cdot yNH_3$	Ammonium Iron (III) Citrate (Ferric Citrate)	Sigma F5879	1185-57-5	265.0
$FeCl_3 \cdot 6H_2O$	Iron (III) Chloride Hexahydrate	Sigma **236489**	10025-77-1	270.3
$MnCl_2$	Manganese (II) Chloride Tetrahydrate	Sigma Aldrich M5005	13446-34-9	197.9
$C_{12}H_{22}O_{11}$	Sucrose Grade II	Sigma S5391	57-50-1	342.3

MW: Fresh Molecular Weight

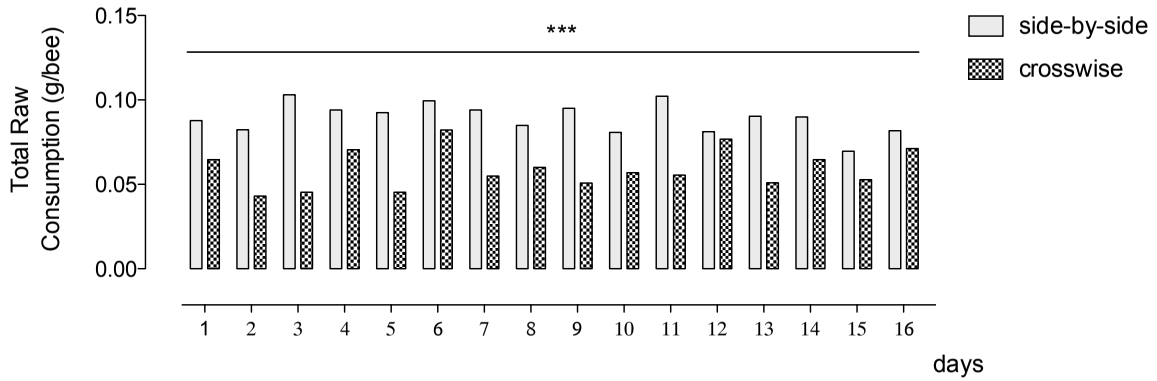
S2 Figure. Adult worker bees found dead during feeding experiments. These two bees were removed from the same treatment box and had the same age at the time of death (collected as newly emerged bees). Bees were weighed and showed 1.5-fold difference in fresh weight (left: 0.153 g: right: 0.094 g). These bees are depicted as they show signs of "unusual looks" and bad physical shape: abrasion and hair loss and possible constipation (refer to Chapter 2).

S3 Figure. Temperature and % humidity records inside incubator chambers used to maintain honey bees during feeding experiments.

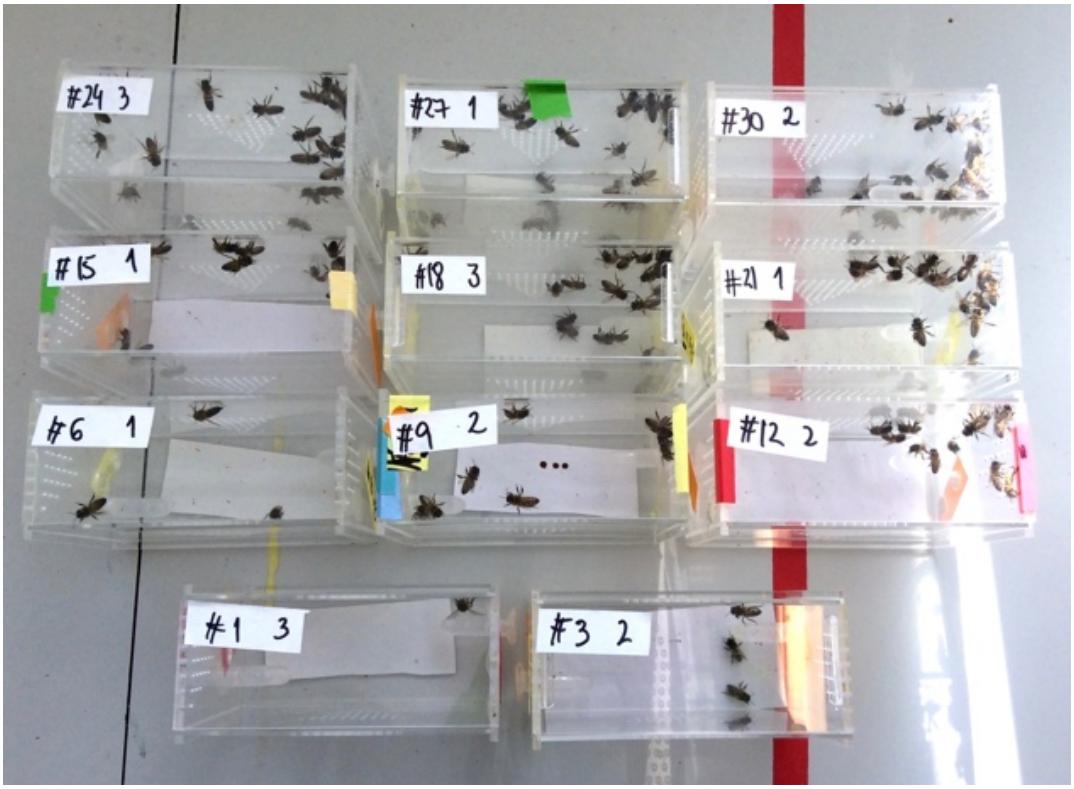


S3 Figure. Monitoring of incubator chambers conditions. Each column of panels respect one incubator. Each row of panels indicate monitoring for each season of feeding experiments. Monitoring is incomplete for year 2015 which is missing recordings from July to September. Year 2016 is complete and covered the whole season of experiments (refer to Chapter 2 and 4).

S4 Figure. Effect of feeding tubes' position on the magnitude of diet consumption over time in choice feeding assays using honey bees.

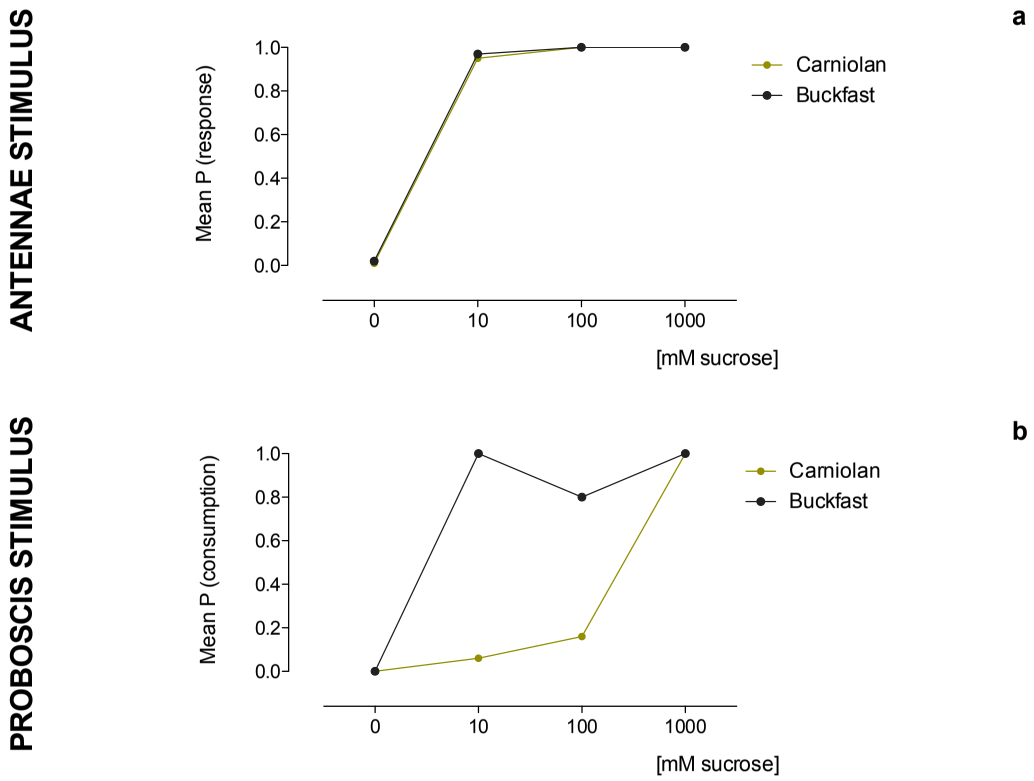


S4 Figure. Testing feeding tubes' position on total solution consumption magnitude over time. Adult bees were offered a choice between sucrose (1.0 M) and sucrose laced with salt over 16 days. Distilled water was also provided. Feeders consisted of modified microcentrifuge tubes with 3—4 holes drilled lengthwise (∅ 2 mm) to allow lapping. Each diet was delivered in two tubes (~5 mL/diet) every day. Two treatment designs varying in tube position were tested: side-by-side or crosswise. In side-by-side regime, diet tube pairs (e.g. sucrose or salt) were shifted across the box every day, whereas in crosswise treatment, diet tubes were counterbalanced in a fixed position (Figure 2.10 a, Chapter 2). Each treatment included N= 7 boxes with N= 30 bees/box. Consumption was recorded by solution change in weight and divided by the number of bees per box each. Bar plot depicts the total (raw) consumption (g/bee) each day for both treatments. Consumption data here were not corrected for evaporation (Evaporation Correction Method A - raw consumption - in section 2.3) nor density. Factorial GEE model was performed to test the effects of box, days and tube position treatments on feeding responses (days x box x tube position: χ^2= 2,978. x 10^{12}, df= 30, P< 0.001; days x box: χ^2= 7,030. x 10^{10}, df= 31, P< 0.001; days x tube position: χ^2= 52.8, df= 15, P< 0.001; box x tube position: χ^2= 1.70, df= 6, P= 0.95). *** significant differences at 0.05% (refer to Chapter 2).



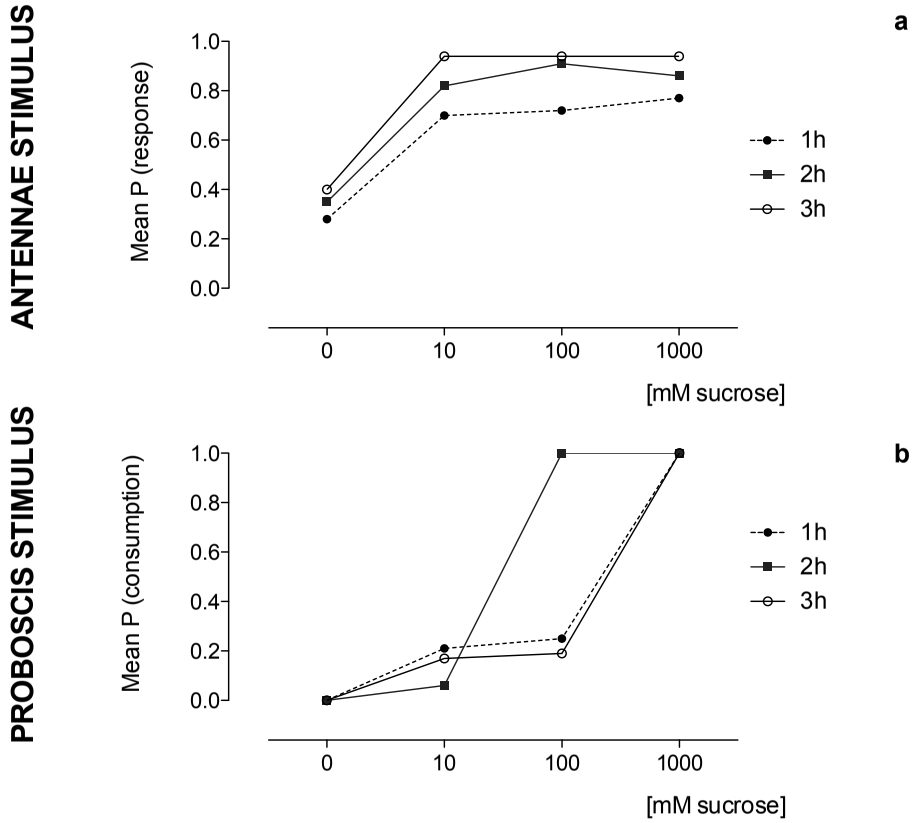
S5 Figure. Experimental set up to test the effects of number of adult honey bees on the reliability of sucrose solution consumption measurements (refer to Figure 2.11, Chapter 2).

S6 Figure. **Sucrose responsiveness of forager honey bees from two bee stocks (carniolan vs. buckfast) stimulated either on the antennae or the proboscis with increasing concentrations of sucrose solutions.**



S6 Figure. Proboscis extension reflex (PER) responses after stimulation on the antennae (a: carniolan, N=5 7/treatment; buckfast, N= 60/treatment) or the proboscis by feeding (b: carniolan, N= 18—20/treatment; buckfast, N= 20/treatment). Stimulus consisted of increasing concentrations of sucrose solutions (0 (water) - 1,000 mM). In **panel a** is shown the mean probability of bees eliciting PER to each solution upon antennal stimulation. GEE: bee.stock x conc: χ^2= 0.14, P= 0.99; bee.stock: χ^2= 0.04, P= 0.84; conc: χ^2= 97.9, P< 0.001. **Panel b** shows honey bee drinking responses upon proboscis stimulation as the mean probability of bees that consumed the whole droplet (4 µL) of each solution. No control solution (distilled water, 0 mM sucrose) was used to stimulate the proboscis, but here was depicted as zero for visualization. GzLM: bee.stock x conc: χ^2= 7.93, P< 0.05 (refer to Chapter 3).

S7 Figure. **Sucrose responsiveness of forager honey bees stimulated either on the antennae or on the proboscis with increasing concentrations of sucrose solutions after exposure to different starvation times.**

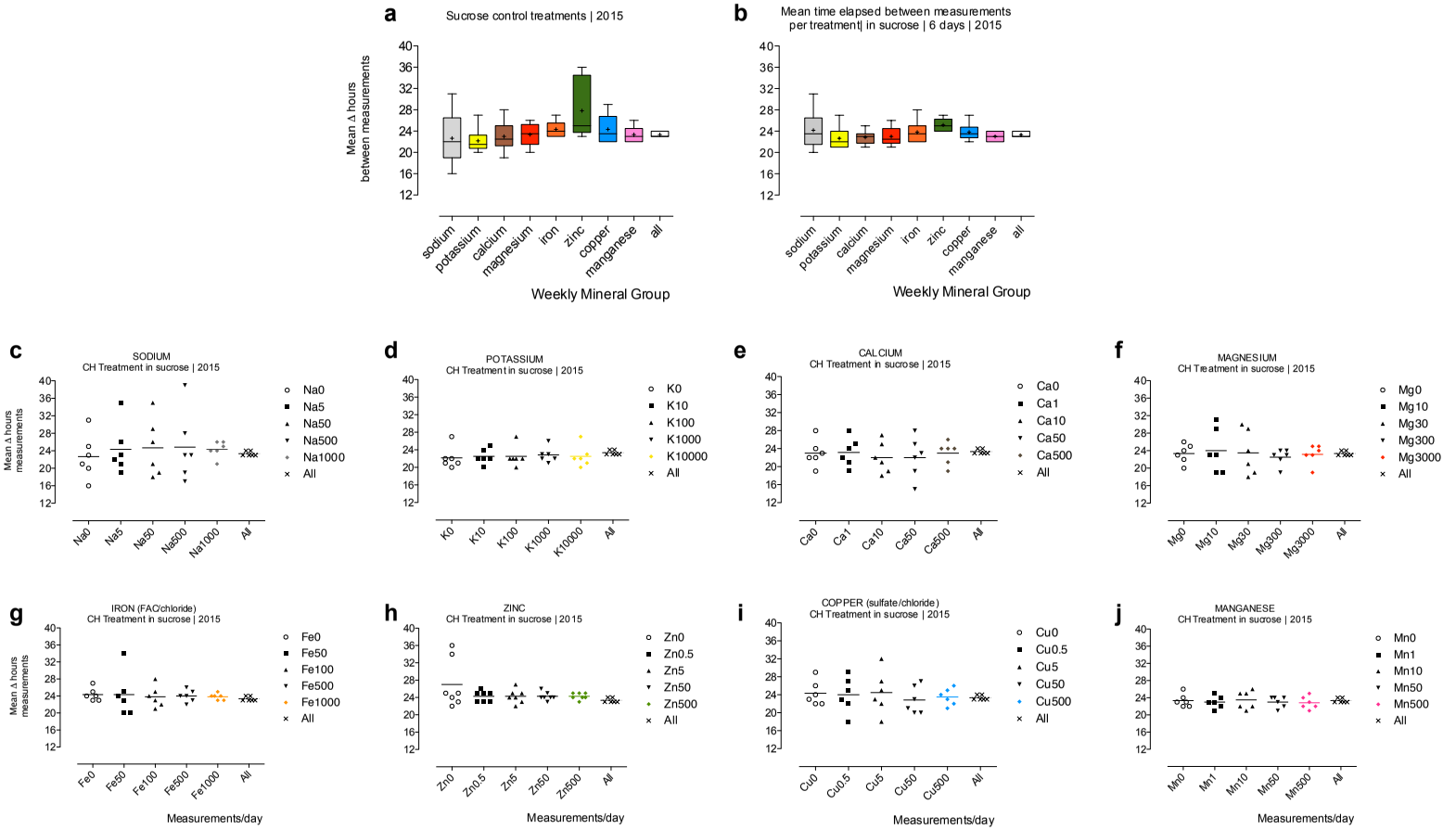


S7 Figure. Proboscis extension reflex (PER) responses after stimulation on the antennae (a: N= 53—57/treatment) or the proboscis by feeding (b: N= 14—20/treatment). Stimulus consisted of increasing concentrations of sucrose solutions (0 (water) - 1,000 mM). In **panel a** is shown the mean probability of bees eliciting PER to each solution upon antennal stimulation. GEE: starv.time x conc: χ^2= 8.52, P= 0.22; starv.time: χ^2= 17.4, P< 0.001; conc: χ^2= 162., P< 0.001. **Panel b** shows honey bee drinking responses to proboscis stimulation as the mean probability of bees that consumed the whole droplet (4 µL) of each solution. No control solution (distilled water, 0 mM sucrose) was used to stimulate the proboscis, but here was depicted as zero for visualization. GzLM: starv.time x conc: χ^2= 13.5, P< 0.001 (refer to Chapter 3).

S8 Table. List Equipment and main materials used in Feeding and Behavioural Experiments (refer to Chapters 2 and 4).

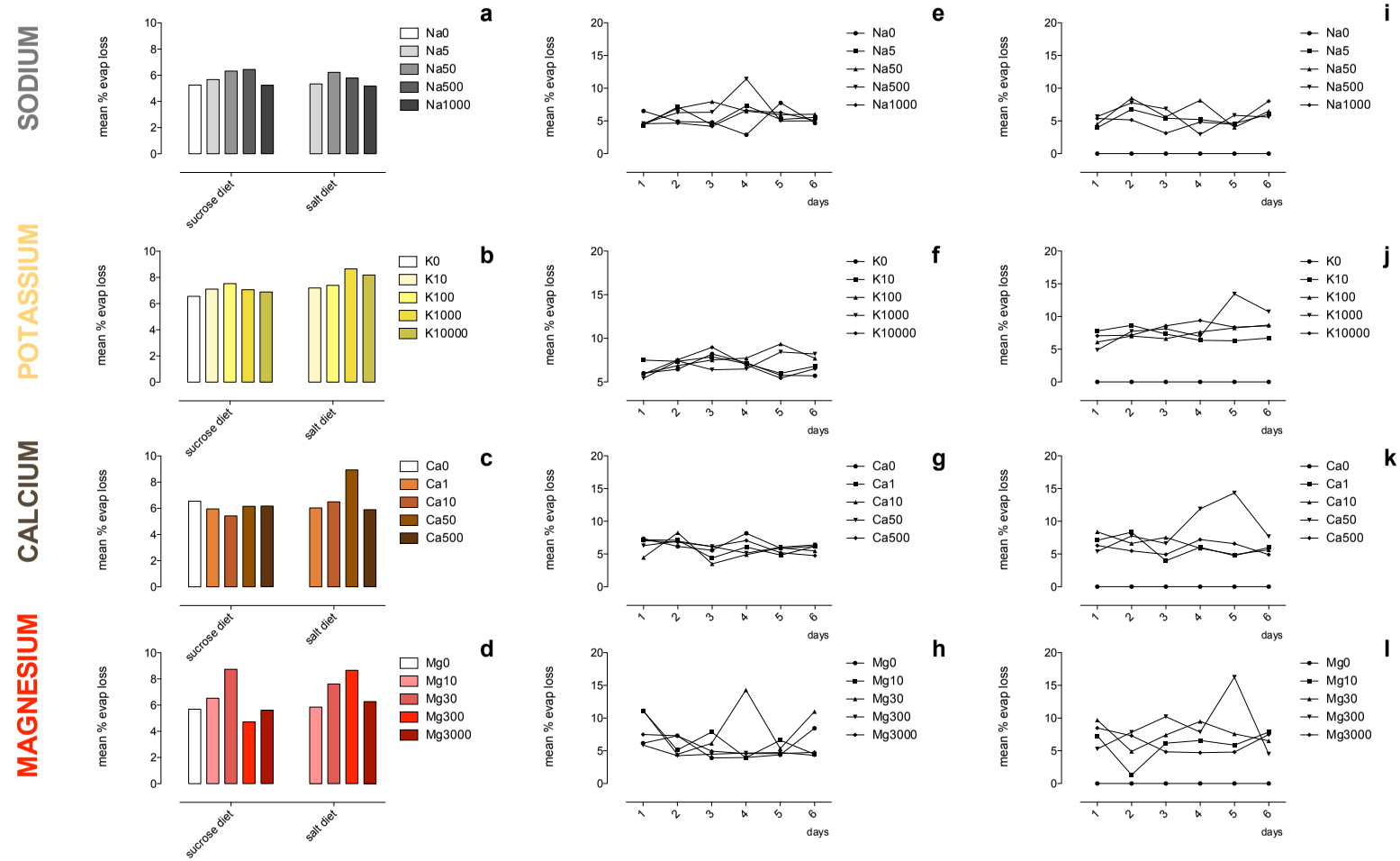
Equipment/Material	Description	Supplier
QUINTIX 64 1S	Analytical Balance, maximum capacity 64g, readability 0.1mg, with Data Cable mini USB USBA (YOO04-D09) for DataLogger	Sartorius UK Limited
Sanyo MIR-553	Heated and Refrigerated Incubator Chamber	Sanyo, UK
pH meter	Accumet Basic AB15 PH meter	Fisher Scientific
OM-EL-USB, Portable Data loggers	Series for Temperature and Humidity; Lithium batteries, EVE brand, er14250, 1/2AA 3.6V	OMEGA Engineering Inc., UK
Centrifuge tubes, 50 ml	Centrifuge tubes 525-0160 PP, 9400×g, non-sterile (Diet Storage)	VWR International
Eppendorf Microcentrifuge tubes	Safe-Lock, 2.0mL Transparent (Feeding Tubes)	VWR International

S9 Figure. Time elapsed between daily consumption measurements in feeding assays using young bees.



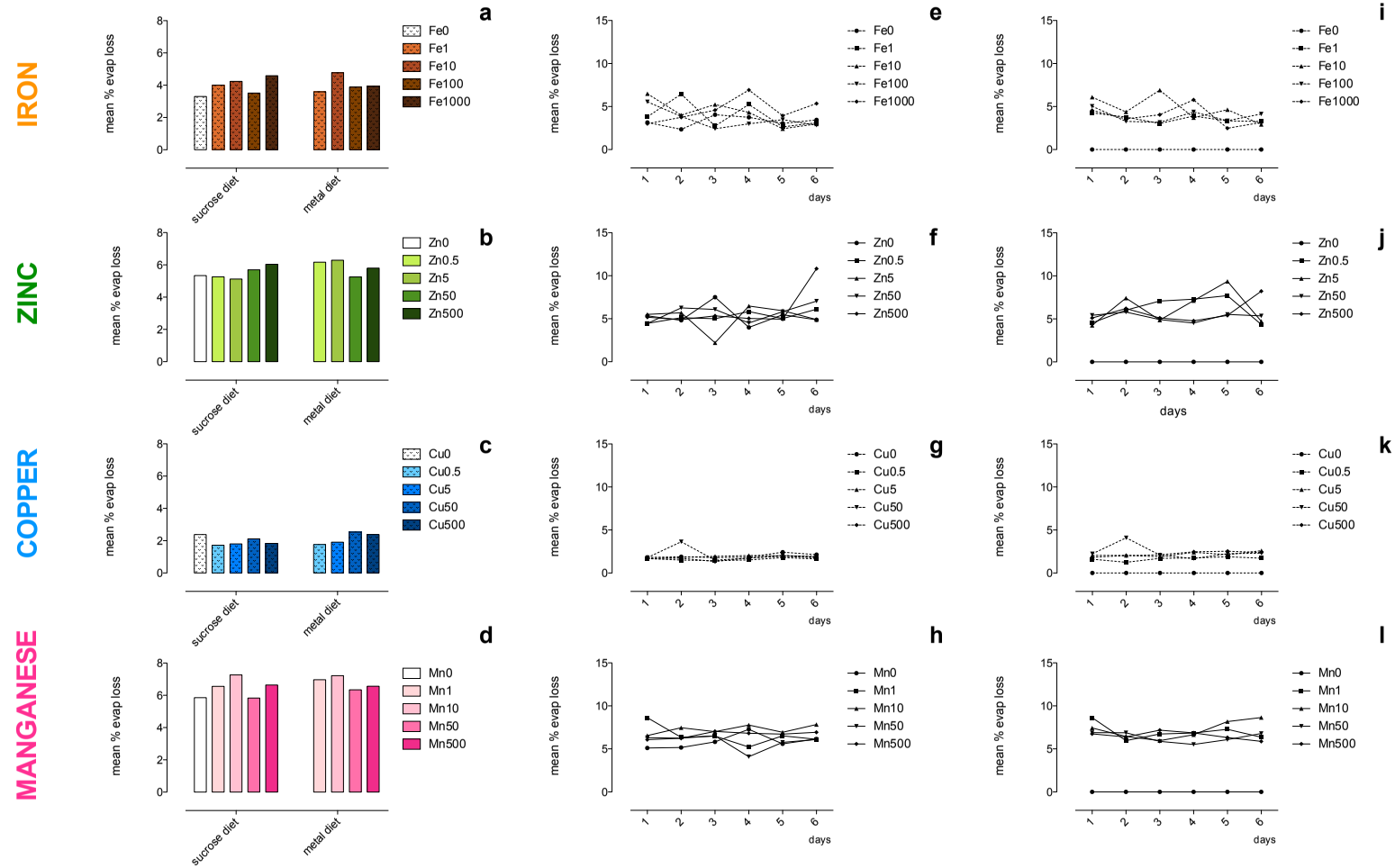
S9 Figure. Time elapsed between consumption measurements in feeding assays over 6 days. One mineral group (e.g sodium) was conducted per week (6 days) across the season (2015). Panels a and b represent time elapsed between daily measurements. **a)** depicts control treatments only in each week (mineral group) and **b**) respects all treatments in each week (for each mineral group). Box-plots indicate the minimum and maximum range of values for each independent treatment, (—) and (+) indicate median and mean, respectively. Each mineral salt is represent by its attributed color code. Scatter Plots indicate mean (—) time elapsed per treatment for each salt (c—f) or metal group (g—j) (refer to Chapter 4).

S10 Figure. Mean % solution loss from salt mock diets used in feeding assays to control for evaporation in Chapter 4.



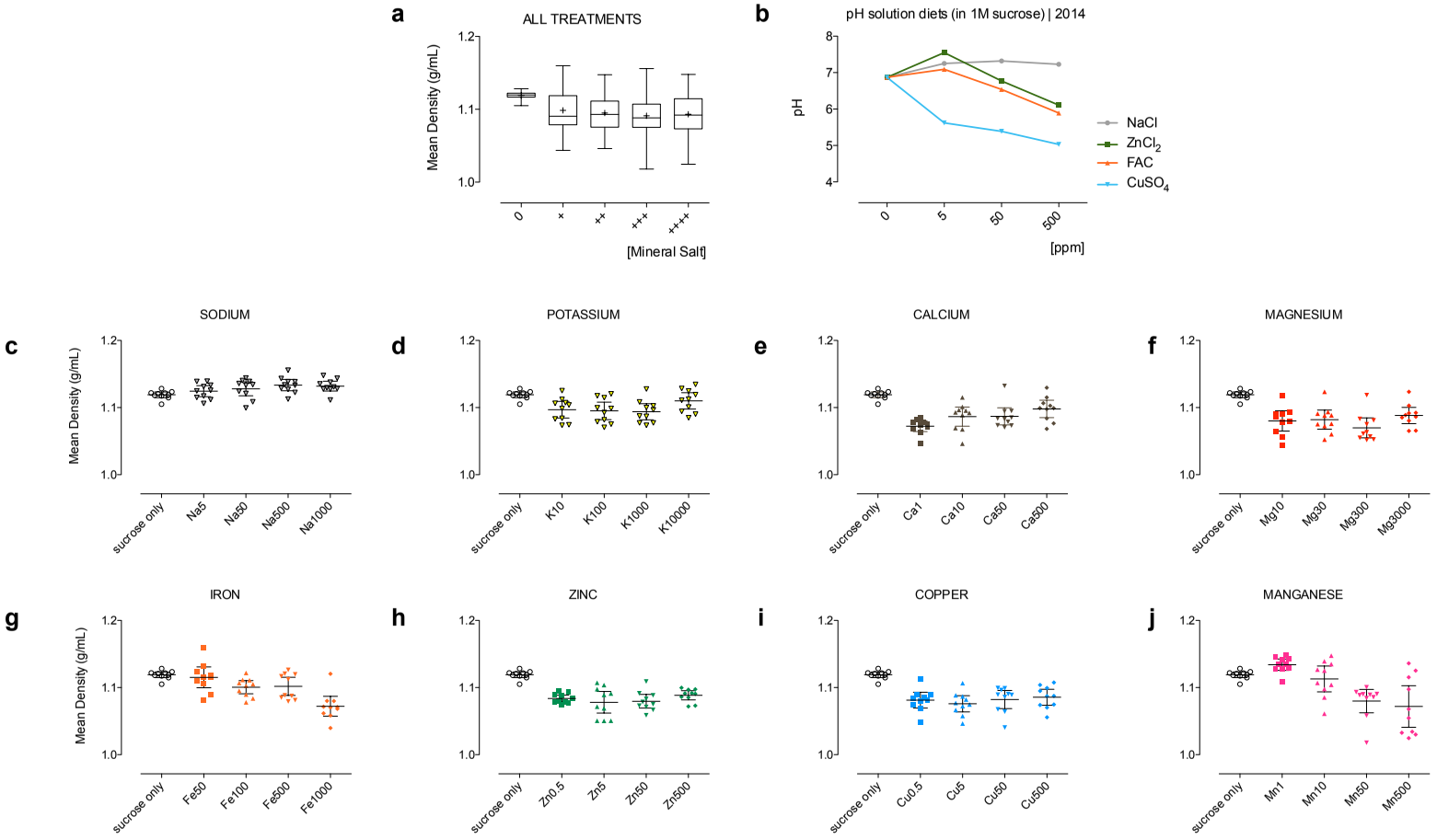
S10 Figure. Mean % solution loss from salt diets in mock evaporation boxes used in feeding assays. **Panels a—d** show the mean % loss per diet treatment. **Panels e—h** show daily mean % loss per sucrose paired diet per treatment. **Panels i—j** show daily mean % loss per salt diet per treatment. Calculations and methodologies are detailed in Chapter 2. Values indicated per diet treatment in panels a—d were used to correct consumption data for evaporation loss (refer to Chapter 4).

S11 Figure. **Mean % solution loss from metal mock diets used in feeding assays to control for evaporation in Chapter 4.**



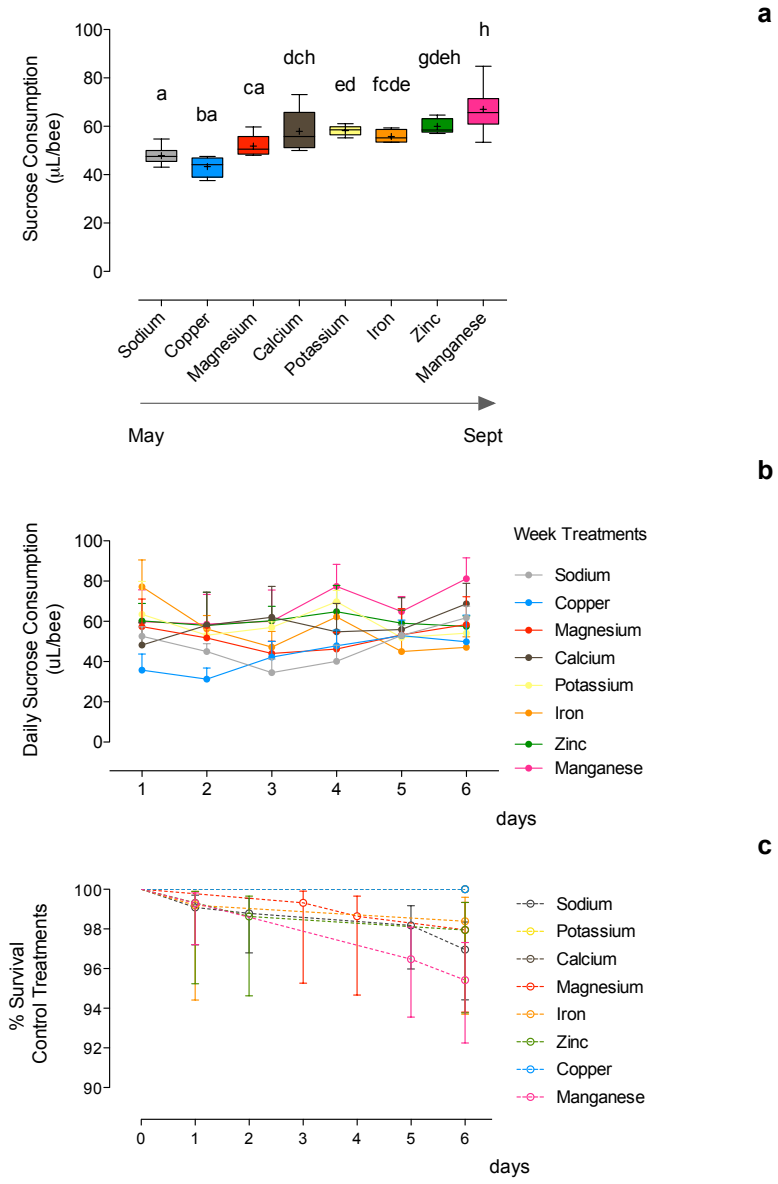
S11 Figure. Mean % solution loss from metal diets in mock evaporation boxes used in feeding assays. **Panels a—d** show the mean % loss per diet treatment. **Panels e—h** show daily mean % loss per sucrose paired diet per treatment. **Panels i—j** show daily mean % loss per metal diet per treatment. Calculations and methodologies are detailed in Chapter 2. Values indicated per diet treatment in panels a—d were used to correct consumption data for evaporation loss (refer to Chapter 4).

S12 Figure. Experimental densities of treatment solutions used in feeding assays in Chapter 4.



S12 Figure. Mean density of treatment solutions per mineral salt. Densities were acquired by weighing 1 mL of each solution (at room temperature) 10 times. **Panel a** represents pooled densities of mineral solutions per level of concentration (0 to high, ++++). Box-plots indicate the minimum and maximum range of values for each independent treatment, (—) and (+) indicate median and mean, respectively. **Panel b** indicates the measured pH for different mineral salts. **Panels c—f** and **g—j** show the density of treatment solutions (mean± 95% CI) for each salt or metal, respectively. Each mineral salt is represent by its attributed color code (refer to Chapter 4).

S13 Figure. Sucrose solution consumption and survival in control cohorts using young worker bees in Chapter 4.



S13 Figure. Sucrose solution (1.0 M) consumption and survival in control treatments (no-choice). Every mineral treatment included one control treatment consisting of sucrose only solution (no salts/metals added). Panels a and b indicate sucrose consumption by control bees for each mineral group (2015/2016). **a)** Indicates the total consumption across the season (May to September). Box-plots indicate the minimum and maximum range of values for each independent treatment; (—) median; (+) mean. One-way GzLM: mineral identity: χ^2= 148., df= 7, P< 0.001. **b)** Represents the daily consumption in control treatments over 6 days for each mineral group conducted in subsequent weeks. **c)** Shows bee survival in control sucrose treatments for each mineral treatment. Kaplan-Meier Tests analysed differences between survival curves. Overall comparisons across 6 days: Log-Rank, χ^2= 19.5, df= 7, P< 0.01; Breslow, χ^2= 19.4, df= 7, P< 0.01; Tarone-Ware, χ^2= 19.5, df= 7, P< 0.01. Different letters indicate (pairwise) statistical significance between group means at 5% (P< 0.05) (refer to Chapter 4).

S14 Table. One-way Generalized Linear Models (GzLM) testing the effect of salt/metal concentration on the feeding responses of young worker bees under two-choice feeding assays over 6 days. Analysis of diet volume consumption (μL/bee) as shown in Figures 4.7 (salts) and 4.8 (metals). Each treatment was measured independently. Salt and metal treatments were analysed independently (Chapter 4).

Source	Wald $\chi 2$	df	P value
Salt Treatments			
Salt Diet			
Sodium	2,442.	4	**<0.001**
Potassium	5,675.	4	**<0.001**
Calcium	2,346.	4	**<0.001**
Magnesium	1,236.	4	**<0.001**
Total Diet			
Sodium	64.1	4	**<0.001**
Potassium	7.49	4	0.11
Calcium	12.3	4	**0.02**
Magnesium	23.5	4	**<0.001**
Metal Treatments			
Metal Diet			
Iron	2,528.	4	**<0.001**
Copper	997.	4	**<0.001**
Zinc	3,563.	4	**<0.001**
Manganese	1,499.	4	**<0.001**
Total Diet			
Iron	22.8	4	**<0.001**
Copper	5.97	4	0.20
Zinc	371.	4	**<0.001**
Manganese	5.55	4	0.24

Values in bold highlight a probability value (P value) < 0.05, indicating a mean difference significant at the level of 5%. Non-significant higher order interactions were removed from the model in a stepwise manner

S15 Table. Survival table for the effects of mineral salt feeding treatments on the survival of young worker bees under two-choice assays conducted over 6 consecutive days as shown in Figure 4.11 in Chapter 4.

Mineral Treatment	Level	Diet	N (boxes)	N (total bees)	N (censored bees)*	Survival (%)
Sodium	0	Na0	10	322	312	96.9%
	+	Na5	9	324	314	96.9%
	++	Na50	10	315	311	98.7%
	+++	Na100	10	314	311	99.0%
	++++	Na1000	10	301	299	99.3%
	Overall			**1576**	**1547**	**98.2%**
Potassium	0	K0	5	151	157	100.0%
	+	K10	10	306	302	98.7%
	++	K100	10	306	304	99.3%
	+++	K1000	10	311	309	99.4%
	++++	K10000	10	301	300	99.7%
	Overall			**1375**	**1366**	**99.3%**
Calcium	0	Ca0	5	138	138	100.0%
	+	Ca1	10	310	310	100.0%
	++	Ca10	10	318	317	99.7%
	+++	Ca50	10	309	306	99.0%
	++++	Ca500	10	310	309	99.7%
	Overall			**1385**	**1380**	**99.6%**
Magnesium	0	Mg0	5	148	145	98.0%
	+	Mg10	10	299	284	95.0%
	++	Mg30	10	303	301	99.3%
	+++	Mg300	10	300	288	96.0%
	++++	Mg3000	10	305	274	89.8%
	Overall			**1355**	**1294**	**95.4%**
Iron	0	Fe0	4	124	122	98.4%
	+	Fe1	**7**	220	209	95.0%
	++	Fe10	**7**	178	175	98.3%
	+++	Fe100	**7**	188	184	97.9%
	++++	Fe1000	**7**	189	177	93.7%
	Overall			**899**	**867**	**96.4%**
Copper	0	Cu0	4	138	138	100.0%
	+	Cu0.5	6	202	198	98.0%
	++	Cu5	6	225	224	99.6%
	+++	Cu50	6	205	195	95.1%
	++++	Cu500	6	223	131	58.7%
	Overall			**993**	**886**	**89.2%**
Zinc	0	Zn0	**5**	146	143	97.9%
	+	Zn0.5	10	303	292	96.4%
	++	Zn5	10	307	298	97.1%
	+++	Zn50	10	299	288	96.3%
	++++	Zn500	10	310	280	90.3%
	Overall			**1365**	**1301**	**95.3%**
Manganese	0	Mn0	10	284	271	95.4%
	+	Mn1	10	287	278	96.9%
	++	Mn10	10	288	285	99.0%
	+++	Mn50	10	282	271	96.1%
	++++	Mn500	10	295	283	95.9%
	Overall			**1436**	**1388**	**96.7%**

*Right-censored bees: live bees at the end of the experiment.

S16 Table. Testing the Proportionality of Hazards assumption by fitting a univariate Cox model with time-by-covariate interactions. Hazards are constant overtime if P value > 0.05. Estimated mean hazard ratios (HR = Exp(ß)) with 95% confidence intervals (95% CI) and P values for model covariates are given (refer to Figure 4.11 in Chapter 4).

Source	Variables in the Model: Risk factors	Mean HR[1] (Exp (ß))	95% CI Lower	95% CI Upper	P value
Salts					
Sodium	time x concentration	0.92	0.78	1.08	0.30
	concentration	0.58	0.77	12.2	0.64
Potassium	time x concentration	3.09	n/a[2]	n/a	0.11
	concentration	n/a	n/a	n/a	0.95
Calcium	time x concentration	n/a	n/a	n/a	0.73
	concentration	n/a	n/a	n/a	0.72
Magnesium	time x concentration	0.95	0.85	1.06	0.39
	concentration	11.6	1.24	109.	**0.03**
Metals					
Iron	time x concentration	1.04	0.91	1.19	0.59
	concentration	2.00	0.11	36.5	0.64
Copper	time x concentration	1.12	0.88	1.43	0.35
	concentration	n/a	n/a	n/a	**<0.01**
Zinc	time x concentration	0.99	0.90	1.10	0.89
	concentration	5.49	0.66	45.4	0.11
Manganese	time x concentration	0.95	0.87	1.05	0.33
	concentration	1.93	0.34	11.1	0.46

[1]HR: Hazard Ratio (mean relative risk); time: time-dependent covariate; concentration was used as a categorical factor with 4 levels. [2]n/a: not applicable because model did not converge, and test values very low. P value < 0.05 is considered significant at the level 5% and highlighted in bold.

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The only good thesis,
is a finished written thesis

She sings, poor reaper,
Thinking herself happy perhaps;
She sings and she reaps and her voice, full
Of joyful and anonymous widowhood,

Quaver like the song of a bird
Limpid as s threshold in the air,
And there are curves in the gentle story
Of the song that she has to sing.

To hear her delights and saddens,
In her voice there is field and labor,
And she sings as if she had
More reasons to sing that life itself.

Ah, sing, sing without reason!
What in me feels is thinking.
Pour into my heart
Your uncertain, quavering voice!

Ah, to be able to be you, being me!
To have your joyful unconsciousness,
And the consciousness of it! Oh heaven!
Oh field! Oh song! Science

Weighs so much and life is so brief!
Enter into me! Turn
My soul into your lofty shadow!
Then, carrying me away, pass on!

by Alberto Caeiro, Heterónimo
Fernando Pessoa
from "She Sings, Poor Reaper"
In Cancioneiro

Ela canta, pobre ceifeira
Julgando-se feliz talvez;
Canta, e ceifa, e a sua voz, cheia
De alegre e anónima viuvez,

Ondula como um canto de ave
No ar limpo como um limiar,
E há curvas no enredo suave
Do som que ela tem a cantar.

Ouvi-la alegra e entristece,
Na sua voz há o campo e a lida,
E canta como se tivesse
Mais razões p'ra cantar que a vida.

Ah! canta, canta sem razão!
O que em mim sente 'stá pensando.
Derrama no meu coração
A tua incerta voz ondeando!

Ah, poder ser tu, sendo eu!
Ter a tua alegre inconsciência,
E a consciência disso! Ó céu!
Ó campo! Ó canção! A ciência

Pesa tanto e a vida é tão breve!
Entrai por mim dentro! Tornai
Minha alma a vossa sombra leve!
Depois, levando-me, passai!

por Alberto Caeiro, Heterónimo
Fernando Pessoa
em "Ela Canta, Pobre Ceifeira"
In Cancioneiro