Università degli Studi di Sassari Dipartimento di
Medicina Veterinaria
Alta Formazione Scuola di Dottorato di Ricerca

Indirizzo: Curriculum Qualità e Sicurezza alimentare

CICLO: XXXV

Evaluation of the nutritional status of worker honey bees (*Apis mellifera ligustica* S., 1806) across temporal patterns through morphological analysis

Candidate: Dott. Stéphane Knoll

Supervisor: Prof. Maria Grazia Cappai

Coordinator: Prof. Fiammetta Berlinguer

Esame Finale 2023

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Preface

The following dissertation is written in a “*thesis by publication*” format and comprises of three scientific articles published in international journals.

Note: papers are possibly still under peer review at the time of submission of this dissertation.

The major goal of this dissertation is to explore honey bee nutrition and hive dynamics in a southern temperate climate (Mediterranean: Sardinia, Italy) through selected, easy-to-use, morphological markers of health and nutrition related to the phenotypic plasticity of honey bees. Research efforts reported here aim to test novel hypotheses involving honey bee nutrition and possible shifts in environmental factors (climatological conditions and plant phenology) in the context of global colony losses in this age of climate change. A specific focus is put on the seasonal bimodal distribution of honey bees; the transition between their summer and winter state, as winter represents a period of particular vulnerability for colonies in temperate climates of the northern hemisphere with fundamental physiological processes underlying survival dictated by nutrition.

The main questions were:

1) Can robust measurements of individual size be used for the assessment of the nutritional status of honey bees?
Hypothesis: The fundamental physiological and nutritional differences between worker honey bee types (Nurse *Vs.* Forager bees and Summer *Vs.* Winter bees) result in measurable morphological differences.

2) What are the effects of present climatic conditions on the seasonality of Italian honey bees in a southern temperate climate (Mediterranean: Sardinia, Italy)?
Hypothesis: High ambient temperatures and current plant phenology are enabling honey bees to remain active year-round.

3) What are the effects of environmental factors on the nutritional status of Italian honey bee colonies in a southern temperate climate (Mediterranean: Sardinia, Italy)?
Hypothesis: Climatic factors in summer as well as winter (indirectly) cause nutritional stress for honey bee colonies in the Mediterranean by affecting both plant and honey bee phenology.

In order to answer these questions, work was divided into two research tasks:

Task 1 consisted of an extensive literature review of the current knowledge on honey bee seasonal physiology and hive dynamics.

Task 2 was based on the monitoring of honey bee workers through morphological and biochemical metrics as a representation of their nutritional health status.

Monitoring activities were performed on *A. mellifera ligustica* colonies housed in two apiaries in Sassari, Sardinia, over a period of 3 years.

This dissertation entitled "***Evaluation of the nutritional status of worker honey bees (Apis mellifera ligustica*** **S.*, 1806) across temporal patterns through morphological analysis*"** is divided in 5 chapters:

CHAPTER 1: Consist of a general introduction, providing the necessary background for this dissertation.

CHAPTER 2: Includes a review article entitled "***The seasonality of honey bee (Apis mellifera L., 1758) colonies. Highlights on summer to winter bee transition and the mechanism behind it. A review"*** published in the journal *Livestock Science* (2020). This chapter consists of an in-depth review of honey bee worker dynamics, division of labour, and aging across temporal patterns. A specific focus is put on the bimodal seasonal transition of honey bee colonies between their summer and winter state and the complex mechanism and driving forces behind this unique adaptation.

CHAPTER 3: Includes an original research article entitled "***Preliminary evaluation of selected morphological metrics for honey bee nutrition and health assessment*"** submitted for peer review in 2023. This manuscript reports on the preliminary assessment of selected morphological measurements as possible markers for the nutritional and health status of *A. mellifera ligustica* forager bees through correlation analysis. Additionally, as a proof of concept, selected metrics were employed in the nutritional monitoring of forager bees over the transition period of autumn-winter-spring (2019-2020) in order to detect the presence of winter bees.

Manuscript under review at the time of submission of this dissertation.

CHAPTER 4: Includes an original research article entitled "***The nutritional year-cycle of Italian honey bees (Apis mellifera ligustica) in a southern temperate climate"*** submitted for peer review in 2023. This manuscript reports on the year-round (2022-2023) nutritional monitoring of honey bee workers (both nurse and forager bees) through morphological and biochemical metrics. Temporal variations in the nutritional status of worker bees according to climatological factors and plant diversity are discussed.

Manuscript under review at the time of submission of this dissertation.

CHAPTER 5: Consists of a general conclusion of this dissertation.

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Abstract

Global honey bee colony losses represents a significant and complex issue in modern apiculture. Regardless of climate, most losses occur in winter, which is a particularly challenging period for *Apis mellifera*. In fact, in order to deal with the harsh winter conditions of temperate climates of the northern hemisphere, honey bees evolved a seasonal caste system (summer *Vs*. winter bees) with fundamental physiological processes underlying survival dictated by nutrition. Whereas the overwintering of honey bees in northern regions has been well studied, much less is known regarding the seasonal dynamics of these social insects in southern temperate climates. Because honey bee colony losses are not restricted to cold climates, and nutrition is a key factor in overwintering success, there is a pressing need to deepen our understanding of honey bee seasonality and nutrition in southern latitudes. Furthermore, given the complex aetiology of honey bee colony losses, identification of novel markers of honey bee health and nutrition would prove beneficial in the context of ongoing research and monitoring efforts. Lastly, since a higher survival rate of local subspecies adapted to specific climatic conditions has been shown, knowledge of southern honey bee populations is of increasing interest, especially in the face of accelerated climate change the world is facing in present days.

The various publications in this dissertation add to the current knowledge of these subject areas. Firstly, an up-to-date literature review containing a centralised overview of honey bee worker dynamics, division of labour, and seasonality in temperate climates of the northern hemisphere is presented. A detailed description of the relevant physiology of summer and winter bees illustrates how *A. mellifera* has adapted molecular pathways into an effective social system for the division of labour as well as a bimodal, biannual worker caste system. The mechanism behind honey bee seasonality governed by a multitude of internal and external factors with varying sensitivity is described and research gaps are highlighted. Secondly, the results of the preliminary evaluation and proof of concept for the use of robust measurements of individual size (head width, thoracal width and length, abdominal width and length, and total body length) as possible markers for honey bee health and nutrition are reported. Specifically, abdominal and total body length were identified through correlation analysis as suitable candidate markers in forager bees, while head width showed promise as a marker across temporal patterns. Thirdly, the long-term monitoring of selected individual size measurements of the locally adapted *A. m. ligustica* allowed for the first description of the honey bee nutritional year-cycle in a southern temperate climate. Analysis of data according to sampling date, climate (temperature, precipitation, and daylength), and flower diversity, revealed two critical timepoints for honey bee nutrition: summer and winter dearth. Seasonality was in accordance with our previous findings, with a peak in nutrient storage indicating the short presence of winter bees. The noted importance of precipitation for honey bee nutrition in the study area demonstrates how climate change could pose a threat to European honey bee populations in the future. Finally, size measurements were shown to vary significantly between nurse and forager bees further illustrating the potential value of selected morphological markers.

The findings reported in this dissertation provide insight that could benefit ongoing research efforts and inform management or conservation strategies with the ultimate goal of improving overwintering success and preventing unnecessary colony losses. Present results can furthermore be used as a cornerstone for future research on honey bee nutrition and seasonality in southern temperate climates.

CHAPTER 1

Introduction

1. The western honey bee

The western or European honey bee (*Apis mellifera* Linnaeus, 1758; meaning “the honey-carrying bee”) is an iconic insect from the genus Apis spp. (or “honey bees”) best known for its honey-making abilities (Michener, 2007; Gupta, 2014a). While there are 10-11 species of honey bees recognised to date (Engel, 1999; Michener, 2007; Tihelka et al., 2020), in the context of this dissertation, the term “honey bee” refers to *Apis mellifera* specifically.

As the most widespread bee species, the native range of the western honey bee consists of Europe (up to southern Scandinavia), Africa, the Middle East, and parts of Asia (Ruttner, 1988; Sheppard and Meixner, 2003; Han et al., 2012). These bees live in temperate to warm climates, occupying northern climate zones, mountain ranges, tropics, and even deserts (Gupta, 2014a; Yadav et al., 2017). Nowadays the range of *A. mellifera* has expanded to all habitable corners of the globe following deliberate transport by settlers who valued these insects for their various products and services, leading it to be the most commonly managed bee in the world (vanEngelsdorp and Meixner, 2010; Han et al., 2012; Meixner et al., 2013; Gupta et al., 2014b; Osterman et al., 2021).

The ability of *A. mellifera* to colonise nearly all habitats on Earth and adapt to diverse climatic conditions is an attestation of the remarkable adaptability of the species (Le Conte and Navajas, 2008; Gupta, 2014a; Tihelka et al., 2020). In fact, this gave rise to the approximately 30 locally adapted subspecies (or “races”) spread across its extensive geographic range with specific behavioural and morphological characteristics, including African (group A), north-eastern Africa (group Y), western/northern European (group M), eastern European (group C), and Middle Eastern (group O) *A. mellifera* populations (Ruttner, 1988; Engel, 1999; Sheppard and Meixner, 2003; Han et al., 2012; Gupta, 2014; Wallberg et al., 2014; Chen et al., 2016; Yadav et al., 2017; Yunusbaev et al., 2019; Espregueira et al., 2020).

Extreme climatic conditions and temporal variations in feed recourse availability are believed to have been driving pressures of natural selection behind honey bee diversification (Gupta, 2014; Chen et al., 2016). For example, European populations show adaptation to colder temperate conditions through synchronization of breeding activities with plant phenology, the formation of a thermoregulating winter cluster, and the ability to bridge the winter period by means of an extensive flightless period (Han et al., 2012; Gupta, 2014a; Wallberg et al., 2014). Moreover, bees from warmer temperate climates, e.g. the Mediterranean, are known to decrease activities during hot summer months as well, which is followed by a second activity peak in fall (Gupta, 2014a).

An overall higher survival rate of locally adapted races has been recognized, highlighting the importance of geographic honey bee subspecies diversity currently threatened by economically driven breeding practices and hybridisation (Le Conte and Navajas, 2008; Meixner et al., 2013; Büchler et al., 2014; Niño and Cameron, 2015; Yunusbaev et al., 2019; Requier et al., 2019). Hence, knowledge of local *A. mellifera* populations could prove increasingly important in the face of accelerated environmental and climate change the world is facing today (De La Rúa et al., 2009; Büchler et al., 2014; Wallberg et al., 2014).

1.1. The Italian bee

The Italian honey bee (*Apis mellifera ligustica* Spinola, 1806) is a subspecies from the C lineage with an ancient hybrid origin; *A. m. mellifera* and *A. m. carnica* (Franck et al., 2000; Dall'Olio et al., 2007). This bee is described as light-coloured (which is particularly noticeable in queens), with bright yellow abdominal bands and yellow hairs (Ruttner, 1988; Gupta, 2014). Specific morphometrics for its identification are described in detail by Ruttner (1988).

As its name suggests, this race originates from the Italian Peninsula where its natural distribution ranges from the Alps in the North and the Mediterranean Sea to the South (De La Rúa et al., 2009; Gupta et al., 2014a). However, as one of the most popular and commercially interesting honey bee subspecies, managed colonies of *A. m. ligustica* are kept around the globe (Ruttner, 1988; Dall'Olio et al., 2007; Meixner et al., 2010; Gupta, 2014b; Gupta et al., 2014a). In many countries, intense hybridization has occurred and/or Italian honey bees have replaced local subspecies (Ruttner, 1988; Franck et al., 2000; Jensen et al., 2005; Gupta, 2014a; De La Rúa et al., 2009).

The Italian bee is particularly coveted for its effective pollinator services and excellent honey production. This race is also preferred for its large colony size, low swarming, and gentle nature (Ruttner, 1988; Franck et al., 2000; Dall'Olio et al., 2007; Gupta, 2014a; Halvorson et al., 2021). The favourable characteristics of this bee have been fundamental for the progression of modern beekeeping, making it one of, if not *the* most important honey bee subspecies (Ruttner, 1988).

Overall, *A. m. ligustica* is well adapted to the warm temperate climate of the Mediterranean. These conditions lead to an early but slow start of brood rearing which peaks and remains high until late summer-fall. Continuation of brood rearing during winter, with just a short cessation, is not uncommon. Even though the overwintering ability of *A. m. ligustica* is generally good (even in colder climates), brood rearing during the coldest months can put stress on a colony, requiring larger food stores and resulting in exhausted bees in spring (Ruttner, 1988; Gupta, et al., 2014b). Brood rearing is also maintained during the typical Mediterranean summer drought period (Ruttner, 1988), with

Italian bees having a relatively high thermal tolerance (Kovac et al., 2014). Correspondingly, *A. m. ligustica* overall require higher temperatures and more “favourable weather conditions” for foraging than northern subspecies (Gupta, 2014a). Nevertheless, the Italian honey bee has shown great adaptability to various climatic conditions (cold and warm), resulting in it being the most generally distributed of all honey bee races (Ruttner, 1988; Franck et al., 2000; Dall’Olio et al., 2007; Gupta, 2014a; Kovac et al., 2014).

2. Importance of bees

The honey bee is a highly valued and economically significant production animal (semi-domesticated insect) given the numerous products it provides, including honey, beeswax, pollen, propolis, royal jelly, and bee venom, having multiple nutritional, medical, cosmetic, and industrial uses (FAO, 2020; Tihelka et al., 2020; Patel et al., 2021; Phiri et al., 2022). Nevertheless, the economic worth of honey bee products is trumped by the profound environmental and agricultural importance of this social insect in terms of pollination of wild plants and agricultural crops (De La Rúa et al., 2009; Gallai et al., 2009; vanEngelsdorp and Meixner, 2010; Leonhardt et al., 2013; Potts et al., 2016; Klein et al., 2018; Tihelka et al., 2020).

Indeed, many wild bushes, small trees, and flowering plants are dependent on the services of this generalist pollinator and the loss of bee populations would therefore have severe consequences for the reproductive capacity of flora around the globe (Michener, 2007; Klein et al., 2018).

Likewise, pollinators play a key role in agricultural sustainability and food security in a modern climate (Aizen et al., 2009; Leonhardt et al., 2013; Klein et al., 2018; Espregueira et al., 2020). *A. mellifera* is the main insect pollinator of agricultural crops worldwide and is often considered one of the most important agricultural livestock species (De La Rúa et al., 2009; Cridland et al., 2017). Overall, these insects pollinate roughly 70% of the world’s leading food crops, including most fruits and vegetables, and many fibre and forage crops (Klein et al., 2007; Klatt et al., 2014; Stein et al., 2017; Nath et al., 2023). While insect pollination is not essential for all these plants, the absence of such pollination usually results in lower production yield (lower number of seeds or smaller fruit) (Klein et al., 2007; Aizen et al., 2009; Stein et al., 2017; Nath et al., 2023). An increased crop yield of €1200 per hive and an overall contribution to agriculture of bee pollination exceeding €150 billion per year worldwide has been published (De La Rúa et al., 2009; Gallai et al., 2009). Augmented nutritional value and shelf-life of insect-pollinated crops have also been shown (Klatt et al., 2014). With the ever-increasing amount and value of insect-pollinated crops, it is clear that honey bee pollination services are of vital economic, agricultural, and nutritional importance (Michener, 2007; Aizen et al., 2009; Gallai et al., 2009; Potts et al., 2016).

3. Honey bee and pollinator declines

Concerns about pollinator declines (both wild and managed species) have made headlines since the turn of the century, warming of a “pollinator crisis” (Goulson et al., 2015; Aizen et al., 2022; Nath et al., 2023). Overall, data shows global pollinator insect populations have decreased by 20-40% (Nath et al., 2023). Moreover, bee species diversity has declined an average of 25% worldwide since the 1990s (Zattara and Aizen, 2021) and by more than 50% in parts of Europe since 1980 (Goulson et al., 2015). These figures depict a pressing need for pollinator conservation in order to prevent possible economic, agricultural, and ecological consequences (Michener, 2007; Potts et al., 2010a; Goulson et al., 2015; IPBES, 2019; Zattara and Aizen, 2021).

Whilst it is clear that wild pollinators are experiencing severe declines (Goulson et al., 2015;IPBES 2019; Zattara and Aizen, 2021; Aizen et al., 2022), the situation of managed honey bees is not as clear-cut. Various publications do report declines in managed *A. mellifera* colonies, but these are mostly related to Europe and North America (Ellis et al., 2010; Potts et al., 2010b; 2016; vanEngelsdorp and Meixner, 2010; Goulson et al., 2015; Aizen et al., 2022). In fact, the worldwide number of honey bee colonies has increased by 80-85% over the last 60 years (Osterman et al., 2021; Aizen et al., 2022; Phiri et al., 2022) and has increased or remained stable in most countries (Halvorson et al., 2021). This being said, the number of honey bee colonies per capita has decreased by roughly 20% worldwide over the same time period (Phiri et al., 2022). The rate of increase might thus not be sufficient to keep up with agricultural demands, putting constraints on yield growth and possibly threatening global food security in the future (VanEngelsdorp and Meixner, 2010; Aizen et al., 2022; Mashilingi et al., 2022; Phiri et al., 2022).

General population trends aside, managed honey bees have been experiencing staggering colony losses in the recent past, especially in winter (Ellis et al., 2010; Neumann and Carreck, 2010; vanEngelsdorp and Meixner, 2010; Jacques et al., 2017; López-Uribe et al., 2020; Halvorson et al., 2021; Insolia et al., 2022). This phenomenon has given rise to national and international research and monitoring efforts attempting to explain and prevent large-scale losses. For example, the *COLOSS monitoring honey bee colony losses network* and the *Bee Informed Partnership* together monitor colony losses for up to 40 countries in and outside of Europe, including the United States (Brodschneider et al., 2022; Bruckner et al., 2022). Latest reports (2019-2020), show winter losses of 18.1% (COLOSS), representing a steady loss increase from previous years (Gray et al., 2022). Similarly, pre-emptive results (2021-2022) depict overall colony losses of 39.0% in the United States (Aurell et al., 2022).

In Italy, colony losses are monitored through the nationwide *Honey Bee Monitoring network* ("ApeNet: monitoring and research in beekeeping") established in 2009 (Mutinelli et al., 2010; Porrini et al., 2016). Losses across the country of 19.2% (2009), 12.5% (2012), and 11.6% (2013) have been reported (Porrini et al., 2016). Results from the latest COLOSS survey show losses of 10.7% in Italy, indicating continuation of this downward trend (Gray et al., 2022). Overall, the number of managed honey bee colonies in Italy has seen a slow but steady increase in recent years, presently counting approximately 1.57 million honey bee colonies (Vetinfo, 2023).

4. Factors affecting honey bee colony losses; focus on climate change and nutrition

Although the multitude of (interacting) factors behind honey bee colony losses are not fully understood, bee declines have been coupled with the increasing effects of pests and diseases (e.g. the ectoparasitic mite *Varroa destructor*), pesticide use, environmental contamination, beekeeping practices, loss of genetic diversity, climate change, and agricultural intensification causing habitat and forage biodiversity loss (De La Rúa et al., 2009; Neumann and Carreck, 2010; Goulson et al., 2015; Potts et al., 2016; Steinhauer et al., 2018; Nath et al., 2023). In this complex background, inadequate nutrition is believed to have significant consequences for bee health, contributing to these declines (Naug, 2009; Brodschneider and Crailsheim, 2010; Goulson et al., 2015; Steinhauer et al., 2018; Bocquet and Tosi, 2022; Kim et al., 2022).

Climate change is broadly recognised as one of the largest threats to biodiversity worldwide (De La Rúa et al., 2009; Goulson et al., 2015), and has been identified as a serious menace to the survival of cold-adapted honey bee populations (Chen et al., 2016). In fact, the rise in global temperatures has caused habitat reduction and loss of population size for honey bees in the past (Chen et al., 2016). While the effects of current climatic change on honey bees might not yet be as apparent, rapid change is believed to affect seasonal activity and ranges, and disrupt the life cycles of many species, as already observed for some wild pollinators (Gordo and Sanz, 2006; Hegland et al., 2009; Chen et al., 2011; Kerr et al., 2015; Potts et al., 2016; Sánchez-Bayo and Wyckhuys, 2019; Soroye et al., 2020). Besides, significant advancement in honey bee phenology has been noted in the Mediterranean (Gordo and Sanz, 2005; 2006).

The effects of climate change are not limited to global warming, extreme weather events; storms, floods, and droughts are predicted to increase and are expected to have significant effects on bee populations (Le Conte and Navajas, 2008; Goulson et al., 2015). Besides this, climate change promotes the spread of invasive species, pests, and pathogens which could affect honey bees (Le Conte and Navajas, 2008; Schweiger et al., 2010; Switanek et al., 2017; Cornelissen et al., 2019). Synergetic effects of climate change with other primary stressors (e.g. habitat loss) have also been pointed out (Goulson et al., 2015).

A serious concern is that a changing climate will drive the occurrence of mismatches between honey bees and plant phenology, with possible nutritional consequences (Le Conte and Navajas, 2008; Hegland et al., 2009; Potts et al., 2010a; Schweiger et al., 2010; Goulson et al., 2015). A current example of this is the mismatch between *A. mellifera* and one of its main plant resources on the Iberian Peninsula, which has been accredited to increasing temperatures (Gordo and Sanz, 2005; 2006).

Similarly, climate change and agricultural intensification (which is also coupled with increased exposure to pesticides) are causing habitat disruption and floral shifts resulting in inadequate feed availability for bees (Decourtye et al., 2010; Di Pasquale et al., 2013; 2016; Goulson et al., 2015; Vaudo et al., 2015; Di Marco et al., 2019; Dolezal et al., 2019; López-Uribe et al., 2020; Raven et al., 2021). The immense conversion of land into pastures and for agricultural use (with a specific trend towards monoculture crops) is severely reducing the abundance and diversity of floral resources in present days (Emmerson et al., 2016; López-Uribe et al., 2020; Raven et al., 2021). Furthermore, the nutritional quality of monoculture floral resources (crops) is questionable and may not be adequate to support honey bee populations (Decourtye et al., 2010; Requier et al., 2015; Vaudo et al., 2015; Di Pasquale et al., 2016; Filipiak et al., 2017).

Besides abundance, flower diversity has been shown to be an important factor in honey bee nutrition as different pollen and nectar sources vary significantly in their nutritive value, e.g. protein and mineral contents. Hence, a limited variety of flowering plants (monotonous diet) might not provide a complete and adequately balanced diet for honey bee colonies (Keller et al., 2005; Huang, 2012; Di Pasquale et al., 2013; 2016; Goulson et al., 2015; Vaudo et al., 2015; Filipiak et al., 2017), especially as nutritional requirements vary among casts (Brodschneider and Crailsheim, 2010; Filipiak et al., 2017).

Inadequate nutrition has been shown to have significant effects on individual and colony health and development, including colony size, overwintering success, lifespan, and immunity (Keller et al., 2005a; 2005b; Naug, 2009; Alaux et al., 2010; Brodschneider and Crailsheim, 2010; Huang, 2012; Goulson et al., 2015; Di Pasquale et al., 2016; Filipiak et al., 2017; Dolezal and Toth, 2018; Ptaszyńska et al., 2021; Retschnig et al., 2021). Accordingly, the effects of other stressors, like pesticides and disease are exacerbated by malnutrition (Naug, 2009; Huang, 2012; Di Pasquale et al., 2013; 2016; Vaudo et al., 2015; Potts et al., 2016; Bocquet and Tosi, 2022; Nath et al., 2023).

All in all, because bees face a multitude of simultaneous stressors, it is unlikely a single factor is responsible for honey bee colony losses. Previous paragraphs illustrate the interactive and synergetic nature of many of the honey bee stressors, with nutrition and climate playing an underlying role in the majority of them (Le Conte and Navajas, 2008; Naug, 2009). However, the degree and outcome of stressor interactions are hard to predict and could differ geographically (Neumann and Carreck, 2010; Goulson et al., 2015). Therefore, a better understanding of the interplay between (locally adapted) honey bee subspecies and environmental factors, especially in this age of climate change, is needed in order to combat colony losses in general and insure the sustainability of apiculture (Le Conte and Navajas, 2008; Jacques et al., 2017; Switanek et al., 2017; Nath et al., 2023).

5. Selected biomarkers of honey bee health

A standardised biomarker approach for the quantification of honey bee health is of increasing importance in the context of research efforts evaluating the large diversity of factors influencing the unsustainable degree of colony losses seen in recent days (López-Uribe et al., 2020). Monitoring of honey bee colonies through such markers could furthermore allow for the early identification of impacting stressors and subsequent implementation of measures preventing unnecessary losses. Nevertheless, further efforts are necessary to recognise useful biomarkers of honey bee health, especially across temporal (e.g. seasonal) and spatial variations (Münch et al., 2013; Smart et al., 2016; Alaux et al., 2017; 2018; Ricigliano et al., 2018; 2019; Kunc et al., 2019; López-Uribe et al., 2020).

As honey bee health is not solely defined by the absence of disease or increased mortality, but also by the presence of well-nourished individuals capable of efficiently performing colony tasks and resisting a multitude of stressors (Brodschneider and Crailsheim, 2010), honey bee health measures of interest in the context of this dissertation are closely related to nutrition.

Nutation-related metrics reflect colony demography as a marker of colony health (López-Uribe et al., 2020). As the storage of nutrients in honey bees is task-dependent, with nutrient stores generally decreasing with age and depletion of stores marking the onset of foraging (Amdam and Omholt, 2002; Toth et al., 2005; Toth and robinson, 2005; Ament et al., 2010), workers' nutrient stores serve as a proxy for the age of onset of foraging (López-Uribe et al., 2020). This is of particular importance as the transition to the foraging stage is coupled with a drastic increase in mortality and thus dictates lifespan (Knoll et al., 2020; *see chapter two*).

Measurements of size are some of the most basic and easy-to-use metrics for the evaluation of honey bee health. Indeed, the weight and body size of worker bees are known indicators of their nutritional status (Brodschneider and Crailsheim, 2010; Ament et al., 2011). This is illustrated by the fact that nutritional stress results in workers that are lighter and smaller (Kunert and Crailsheim, 1988; Hoover et al., 2006; Scofield and Mattila, 2015; Wang et al., 2016; Schilcher et al., 2022). Daly et al. (1995) also revealed the effects of nutritional stress on adult size through the analysis of various morphometrics. This being said, size variations between *A. mellifera* workers are generally small (Kerr and Hebling, 1964; Roulston and Cane, 2000; Chole et al., 2019), making the evaluation of nutritional status through visual inspection difficult (Scofield and Mattila, 2015).

Alternatively, the nutritional status of worker bees can be assessed through biochemical analysis, e.g. lipid and protein concentrations (López-Uribe et al., 2020). Logically, fluctuations of these nutritional compounds are correlated to feed availability (Kunert and Crailsheim, 1988). Both lipid and protein contents have been linked to survival in honey bees (Smart et al., 2016; Dolezal et al., 2019; Kunc et al., 2019).

Size estimation of the fat body, the central tissue for nutrient storage (lipids and protein) in honey bees, can also be used as a representation of their nutritional status (Toth et al., 2005; Toth and robinson, 2005; Alaux et al., 2010; Smart et al., 2016; Koubová et al., 2021). Fat body mass has been positively correlated with feed availability and diversity in honey bees (Alaux et al., 2010; 2017), and young (nurse) bees to have higher fat body mass than older (forager) bees (Wilson-Rich et al., 2008). Correspondingly, the fat body is the principal organ for the production of the nutrient storage molecule vitellogenin (Vg) (Amdam and Omholt, 2002; Seehuus et al., 2007; Corona et al., 2007). Vitellogenin has emerged as a dominant biomarker for honey bee health and nutrition (Alaux et al., 2011; 2017; Smart et al., 2016; Ricigliano et al., 2018; 2019; 2021; Wegener et al., 2018; Kunc et al., 2019; López-Uribe et al., 2020; Koubová et al., 2021; Sarioğlu-Bozkurt et al., 2022) and Vg-levels have been linked with performance, phenology, immunity, and overwintering success in honey bees (Smart et al., 2016; Alaux et al., 2017; Ricigliano et al., 2018; 2019; Kunc et al., 2019). Moreover, accumulation of this glycolipoprotein has been accredited to cause longevity in both queen and winter bees (Amdam and Omholt, 2002; Amdam et al., 2004; 2005; Seehuus et al., 2006; Corona et al., 2007; Nelson et al., 2007; Münch et al., 2013), therefore serving as a biomarker for aging (Koubová et al., 2021).

As nutrient storage in adult honey bees is predominantly accomplished through the accumulation of Vg specifically (Amdam and Omholt, 2002), body (and fat body) mass can be considered a reflection of fluctuations in the amount of this molecule (Ricigliano et al., 2019). A positive link between abdominal lipid and Vg contents has also been pointed out (Wegener et al., 2018).

Lastly, hypopharyngeal gland (HPG) size and protein content have been identified as markers reflecting the nutritional status of worker bees (Sagili et al., 2007; Degrandi-hoffman et al., 2010;

Alaux et al., 2011; Di Pasquale et al., 2013; Smart et al., 2016). These paired acinous glands in the head of bees produce royal jelly (brood food) from Vg (Amdam and Omholt, 2002; Amdam et al., 2003; Seehuus et al., 2007) and are therefore well developed in nurse and degraded in forager bees (Amdam and Omholt, 2002; Döke et al., 2015; Ali et al., 2019). Additionally, Vg-titers have been correlated to the size of HPG of workers (Fluri et al., 1982) and variations in Vg-contents of the HPGs of nurse *Vs.* forager bees to correspond to nutritional aspects of task-related demography (Seehuus et al., 2007). Cessation of brood rearing in winter furthermore results in HPG hypertrophy in winter bees which has led to the hypothesis these glands might serve a nutrient storage function similar to that of the fat body (Fluri et al., 1982; Crailsheim, 1990; Amdam and Omholt, 2002; Mattila and Otis, 2007; Degrandi-hoffman et al., 2010).

To conclude, it is worth mentioning that, given the significant (physiological) differences in nutritional status between worker bees of different biological age and seasonality (Knoll et al., 2020: *see chapter two*), variation in nutrition-related metrics should be interpreted taking demography and seasonality of samples into account (López-Uribe et al., 2020). For this reason, selective (uniform age) or at least separate sampling of nurse and forager bees is recommended (Ricigliano et al., 2019; López-Uribe et al., 2020).

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CHAPTER 2

The seasonality of honey bee (*Apis mellifera* L., 1758) colonies. Highlights on summer to winter bee transition and the mechanism behind it. A review.

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Highlights

- The honey bee (*Apis mellifera* L., 1758) is a peculiar production animal following a caste system.
- Worker honey bee tasks are linked to age and season.
- Winter bee transition is based in the regulatory mechanisms controlling juvenile hormone metabolism and the age based division of labour of worker bees.
- Various in- and out-hive factors contribute to the regulation of honey bee seasonal transitions.
- Functionality of colony seasonality was reviewed in the light of the latest knowledge.

Abstract

Beekeeping plays a key role within animal productions since millennia. Honey bee colonies consist mainly out of worker bees which, besides based on their behavioural roles, are divided according to seasonal appearance. In this manner, a distinction is made between classical summer bees and long-lived winter or "*diutinus*" bees. Seasonal transition into their winter form allows honey bee colonies to survive harsh winter conditions through in-hive thermoregulation and by initiating brood rearing for the new cycle. Vitellogenin, the main bee nutritional storage molecule, is central in winter bee physiology and accounts for the typical features associated with *diutinus* bees (high hemolymph and fat body lipid and protein contents, hypertrophied hypopharyngeal glands and longevity). Overall, transition into the winter bee state can be explained by extension of the mechanisms regulating juvenile hormone metabolism and worker bee age-based division of labour, namely; changes in bee nutrient stores, brood microclimate, and colony age demography. Furthermore, substantial evidence has been produced indicating the arrival of winter bees to be strongly correlated to decreasing brood rearing activity in fall. Subsequently, factors regulating the brood rearing pattern of honey bees can be considered key triggers in the appearance of winter bees. In this way, the arrival of winter bees is mainly linked to plummeting pollen supplies in fall. The effect of other triggers have either been categorised as lesser, indirect, or their involvement remains unclear.

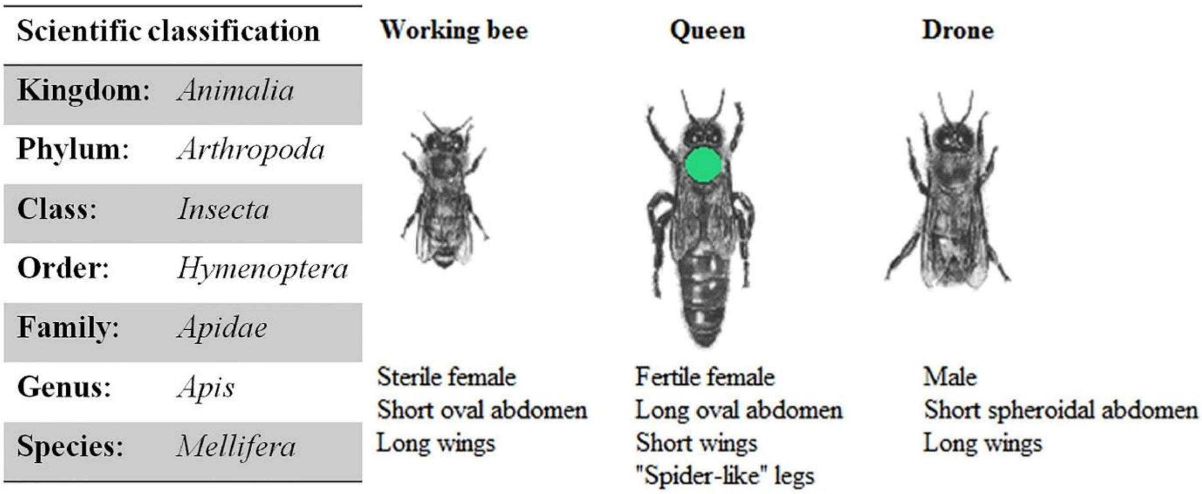
The current knowledge regarding the functionality of the seasonal transition of honey bee colonies has led to the description of an elegant system controlled by a multitude of internal and external factors. Regardless, certain aspects of this phenomenon remain unknown and especially the transition back from winter to summer bees still represents an open question.

KEYWORDS

Fat body, Hypopharyngeal glands, Juvenile hormone, Overwintering, Vitellogenin, Winter bees

1. Introduction

Honey bee (*Apis mellifera* L., 1758) colonies follow a caste system, involving different types of bees (Figure 1), performing different tasks. Firstly, each colony holds one queen, responsible for the reproductive activity within the hive. Secondly, honey bee colonies have a small amount of male bees or drones. Besides that, colonies consist of a large number of sterile females called worker bees (Seeley, 1982; Rueppell et al., 2008; Russell et al., 2013).



Scientific classification	
Kingdom:	*Animalia*
Phylum:	*Arthropoda*
Class:	*Insecta*
Order:	*Hymenoptera*
Family:	*Apidae*
Genus:	*Apis*
Species:	*Mellifera*

Figure 1. Illustration of the different casts of *Apis mellifera*. The green dot on the back of the thorax is used to identify the queen of 2019 within the colony, according to the international queen marking colour.

Worker bees are morphologically identical but can be divided further according to their behavioural roles. Honey bee workers show an age division of labour, where newly emerged bees tend to in-hive tasks, like brood rearing and colony maintenance, and older worker bees tend to tasks outside the hive, like foraging (Seeley, 1982; Huang and Robinson, 1992; 1995; 1996; Leoncini et al., 2004; Toth et al., 2005; Rueppell et al., 2008; Russell et al., 2013; Döke et al., 2015).

Additionally, worker honey bees can be divided into two distinct groups based on seasonal appearance; the classical summer bee and the winter bee or "*diutinus*". Winter bees have been identified within bee colonies living in temperate climates, and just like the name would suggest, this bee-type is present during the colder months of the year (late fall until early spring) (Amdam and Omholt, 2002; Amdam et al., 2005b; Mattila and Otis, 2007; Kunc et al., 2019). Seasonal transition into this winter form allows honey bees, as one of the few insect species, to survive harsh winter conditions, without entering a dormant state (Huang and Robinson, 1995; Döke et al., 2015).

The main basis for the differentiation between the summer and winter bee type is their lifespan. Where summer bees have an average lifespan of 25–40 days, winter bees have been shown to be able to live for over 250 days (Mattila et al., 2001; Amdam and Omholt, 2002; Döke et al., 2015; Kunc et al., 2019; Yamada et al., 2019). Tasks of these long-lived winter bees include regulating in-hive temperatures during winter and the initiation of brood rearing in winter and early spring (Mattila et al., 2001; Mattila and Otis, 2007; Döke et al., 2015; Eyer et al., 2017; Kunc et al., 2019). Besides the evident difference in lifespan, winter bees show pronounced physiological variations compared to summer bees. Overall, winter bees have substantially lower juvenile hormone (JH) titers (Huang and Robinson, 1995; Mattila and Otis, 2007; Döke et al., 2015; Kunc et al., 2019). Juvenile hormone is mostly associated with foraging activities of summer workers and is inversely related to in-hive activities (Huang and Robinson, 1995; Döke et al., 2015). Second, winter bees are known to hold hypertrophied hypopharyngeal glands (HPGs), glands which play a key function in food production (royal jelly) for brood rearing. Next, long-lived winter bees have enlarged fat bodies due to the heavy accumulation of nutrients (lipids and protein) (Mattila et al., 2001; Amdam and Omholt, 2002; Mattila and Otis, 2007; Döke et al., 2015; Kunc et al., 2019). Finally, *diutinus* bees have been shown to have elevated haemolymph protein levels, predominated by vitellogenin (Vg) (Amdam and Omholt, 2002; Döke et al., 2015; Van der Steen et al., 2015; Kunc et al., 2019), a molecule that has been accredited for instigating longevity in queens and winter honey bees (Amdam et al., 2009; Ricigliano et al., 2018; Kunc et al., 2019; Yamada et al., 2019).

Within a colony, transition from the summer to the winter bee state is gradual. As the seasonal cycle progresses towards winter, the proportion of long-lived winter bees will gradually increase with each generation (Mattila et al., 2001; Mattila and Otis, 2007). Although the triggers influencing this transition are not yet fully understood, it is likely that both environmental factors as well as in-hive factors play a role in the matter (Mattila et al., 2001; Amdam and Omholt, 2002; Mattila and Otis, 2007; Döke et al., 2015).

1.1. Annual cycle of the honey bee

In temperate climates, honey bees exhibit an annual brood rearing cycle (Mattila et al., 2001; Döke et al., 2015). Generally, brood rearing begins mid-winter and rises until summer, following the nectar flow. During spring, brood rearing is drastically increased before peaking. In late summer, brood rearing slowly decreases until it (virtually) ceases in late fall (Mattila et al., 2001; Mattila and Otis, 2007; Döke et al., 2015). This strong decrease in brood rearing has evolved as a mechanism to conserve resources in times when foraging is not possible. Anticipation of approaching favourable environmental conditions will spark brood rearing once more (Nürenberger et al., 2018;

Ricigliano et al., 2018).

The high increase in colony size, following the spring rise, typically leads to swarming, where the majority of workers leave the colony together with the queen. The swarm will venture out in search of a new home, leaving behind a new queen with a limited number of workers who will rebuild the old colony (Döke et al., 2015).

From the onset of spring until late fall, bee colonies deploy a large workforce dedicated to the collection of food supplies. As long as the ambient conditions allow it, forager bees will venture out of the hive in search of pollen and nectar. Pollen, the main protein and lipid source of honey bees (Mattila and Otis, 2007; Di Pasquale et al., 2013; Döke et al., 2015), is collected and immediately stored in designated cells for later consumption by young bees (Toth et al., 2005). In turn, nurse bees will convert this protein rich meal into jelly, secreted through their HPGs as feed for the queen (for this reason called royal), the present larvae, and other worker bees (Crailsheim, 1991; Di Pasquale et al., 2013). Nectar, used as a general energy source by bees (Döke et al., 2015), is first collected by foragers before being passed on to in-hive bees through trophallaxis (the exchange of liquids via regurgitation) (Crailsheim, 1991; Leoncini et al., 2004). Following, young bees will pass the gathered nectar between each other before it is stored and converted into honey (Toth et al., 2005; Russell et al., 2013; Döke et al., 2015). Contrary to pollen, honey is often stored for a substantial amount of time before being consumed.

As the annual cycle progresses and brood rearing decreases, hives will make preparations for winter (Mattila and Otis, 2007; Döke et al., 2015). This period is characterized by the exile of drone bees (Langowska and Zduniak, 2019) and the replacement of short-lived summer bees by long-lived winter bees (Mattila et al., 2001; Mattila and Otis, 2007; Döke et al., 2015). During this phase, foraging activities will gradually decrease as a shortening photoperiod and decreasing temperatures restrict any activity outside the hive (Döke et al., 2015; Eyer et al., 2017). Furthermore, colonies are drastically reduced in size, going from up to 70,000 individuals to an approximate colony size of 10,000–20,000 individuals in winter (Van Nerum and Buelens, 1997).

When brood rearing has come to a halt and ambient temperatures fall below 10 °C, winter bees will form a thermoregulating cluster providing isolation and generating heat insuring colony survival (Mattila and Otis, 2007; Döke et al., 2015; Nürenberger et al., 2018). After brood rearing has resumed in mid-winter, the cluster will produce sufficient heat to make sure the brood area retains a suitable temperature to assure proper development of the first generation of worker bees for the new cycle (Döke et al., 2015). Once favourable environmental conditions return, the classical division of labour among bees resumes and winter bees make way for newly immerging summer bees (Mattila and Otis, 2007; Döke et al., 2015).

1.2. Mortality and lifespan

Mortality rates of worker bees differ based on the division of labour, influencing their lifespan. Overall, mortality of in-hive bees is low. Contrasting to this, mortality rates of foragers has been shown to be substantially higher. Foraging exposes bees to high levels of metabolic and oxidative stress. Besides that, predation, weather conditions, and the chance of getting lost pose additional risks to bees venturing outside the hive (Dukas, 2008; Rueppell et al., 2008; Russell et al., 2013). The foraging stage of honey bees lasts about one week and the mortality rate of these bees has been estimated to exceed 15% daily (Russell et al., 2013). Because of the high death rate of foragers and the fact that the time and energy devoted to foraging is relatively constant amongst worker bees, the life expectancy of bees is highly influenced by the age of onset of foraging (Mattila et al., 2001; Rueppell et al., 2008; Amdam et al., 2009; Russell et al., 2013). On average, in-hive worker bees start foraging at about 20 days after eclosion (Russell et al., 2013), but this transition is not static in time (Huang and Robinson, 1992; Amdam and Omholt, 2003; Leoncini et al., 2004; Toth et al., 2005; Rueppell et al., 2008; Russell et al., 2013; Eyer et al., 2017). The shortest possible interval before transition is believed to be around four days of age (Amdam and Omholt, 2003; Russell et al., 2013). The plasticity of worker bee transformation from nurse bee to forager has been shown to be determined by various influences, including in-hive and out-hive factors. Additionally, this transition is believed to be mediated by one central molecule namely: JH [an insect hormone originally identified to prevent the metamorphosis of immature insects (Williams, 1956)]. As JH titers are low in nurse bees and gradually rise until the onset of foraging, this hormone is accredited with regulation of the behavioural development into forager bees (Huang and Robinson, 1992; 1995; 1996; Pankiw et al., 1998; Le Conte et al., 2001; Rueppell et al., 2008; Eyer et al., 2017). Treatment of young bees with JH will artificially induce foraging behaviour and, conversely, foragers who revert to the nursing stage experience a drop in JH (Huang and Robinson, 1992; 1995; 1996). Additionally, structural changes in the brain of honey bees associated with foraging has been accredited to the effects of JH (Huang and Robinson, 1995).

The effect of JH on the division of labour among workers is (at least partly) explained through changes in biogenic amine levels within the brain of bees (Schulz and Robinson, 2001). More specifically, a clear association between octopamine (Op: a well-known neuromodulator) concentrations in the antennal lobes (the primary olfactory processing centres of the brain) of worker bees and their tasks has been found (Schulz and Robinson, 2001; Schulz et al., 2002a). Most likely, this particular amine functions as an activator of foraging by modulation of the responsiveness to task-related stimuli (Schulz and Robinson, 2001; Schulz et al., 2002a; 2002b). Overall, Op levels are low in-hive bees and elevated in foragers. Moreover, this biogenic amine seems to rise prior to the onset of foraging and, just like for JH, experimental treatment of bees with Op induces foraging behaviour (Schulz et al., 2002a). As treatment of worker bees with JH increases brain Op levels and an increase in this amine causes precocious foraging regardless of JH titers, a causal role of Op in foraging initiation can be concluded (Schulz et al., 2002b). This being said, although it is likely the rise in JH preceding the forager transition to initiate the rise in Op, the pathways linking these two molecules still remain unknown. Additionally, Op has been shown to increase the production of JH as well, illustrating a more complex relationship between these two molecules (Schulz and Robinson, 2001; Schulz et al., 2002a; 2002b).

In general, because of the age division of labour among honey bees, the lifespan of workers is regulated to insure sufficient amounts of nutrient stores, enabling brood rearing and survival of the colony year round (Huang and Robinson, 1992; 1996; Mattila et al., 2001; Amdam and Omholt, 2003; Rueppell et al., 2008; Eyer et al., 2017). A general schematic representation of the central function of JH and its interaction with different factors and molecules influencing the nurse to forage transition is depicted in Figure 2.

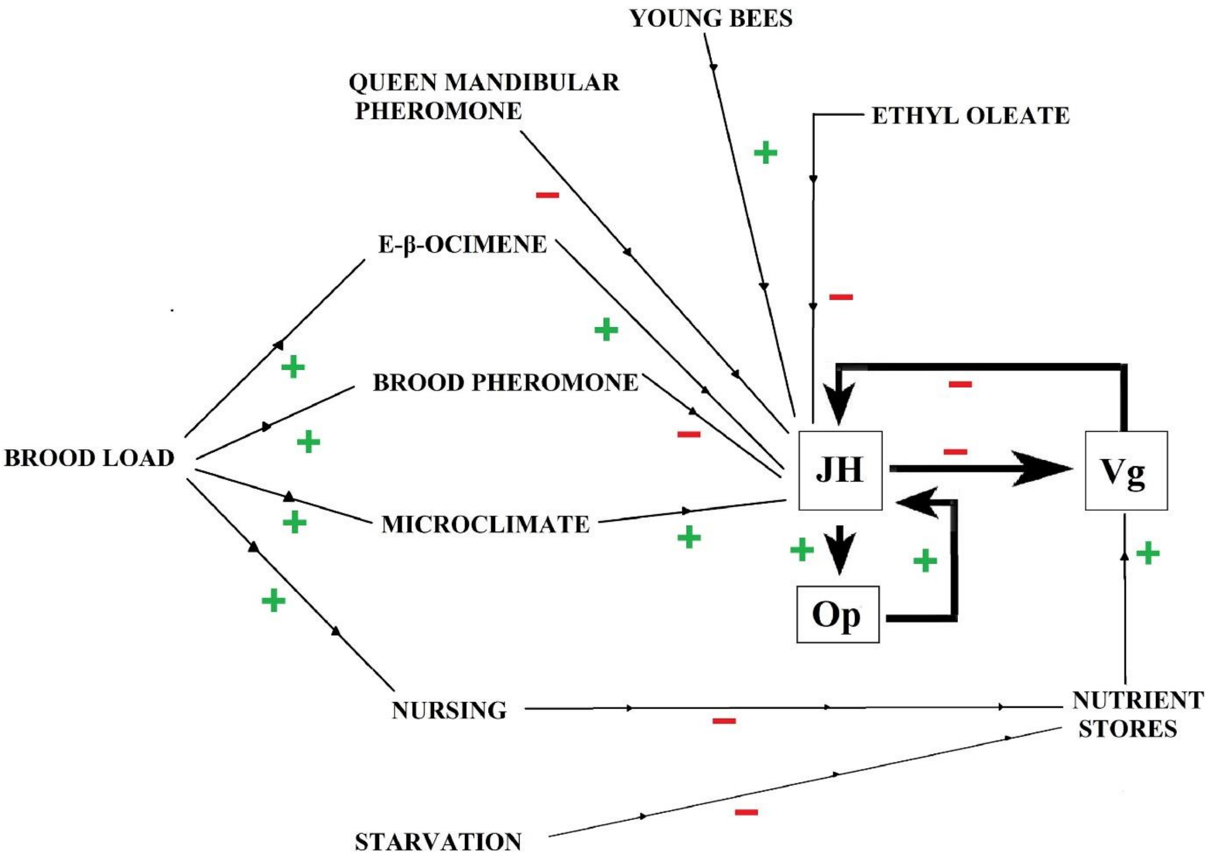


Figure 2. Schematic representation of the interaction between Juvenile Hormone (JH) Vitellogenin (Vg) and Octopamine (Op) in the context of the nurse to forager bee transition and the different factors influencing this transition. The nature of the interaction between each subsequent factor is given by a green “+” or a red “−“ sign in case of positive or negative interaction respectively.

1.2.1. Regulation based on nurse nutrient stores

Firstly, the nutritional status of worker bees is associated with their behavioural roles and seems to play a causal role in the determination of the age of onset of foraging. Overall, forager bees have significantly lower nutrient stores compared to nurse bees. Furthermore, as the act of foraging itself does not have a strong effect on bee nutrient stores [carbohydrates, mostly originating from stockpiled honey, are the only fuel source used for flight activity (Neukirch, 1982; Messan et al., 2018)] and forager bees exhibit exhausted stores from the onset of foraging, nutrient stores of nurse bees thus diminish prior to foraging, possibly signalling the onset of foraging (Amdam and Omholt, 2002; 2003; Toth et al., 2005; Toth and Robinson, 2005; Amdam et al., 2007; 2009).

The mechanisms behind the plasticity of nurse-to-forager transition observed in bees with variable nutrient stores can be explained by the workings of the common insect storage molecule Vg (Amdam and Omholt, 2003; Toth et al., 2005; Rueppell et al., 2008; Amdam et al., 2009). Vitellogenin is the most abundant nutrient storage molecule found in honey bees. In general, Vg synthesis and hemolymph levels are high in nurse bees and low to non-existent in foragers (Amdam and Omholt, 2002; 2003; Amdam et al., 2003; 2004; Eyer et al., 2017).

The glycolipoprotein Vg is very interesting due to its interactions with JH, laying a physiological link between brood load, the nutritive status of worker bees, and longevity. This interaction between JH and Vg has been hypothesized as the main underlying mechanism controlling the transition between the in-hive and foraging stage of worker bees. To this extent, high Vg levels suppress JH and therefore impedes social foraging behaviour, inhibiting the transition to forager. Conversely, a (premature) decrease in Vg accelerates this transition (Amdam and Omholt, 2002; 2003; Toth et al., 2005; Guidugli et al., 2005; Amdam et al., 2007; 2009; Seehuus et al., 2007; Münch et al., 2015). Experimental reduction of Vg in nurse bees will trigger a premature rise in JH (Guidugli et al., 2005) and artificially decreased Vg levels were shown to initiate foraging and cause death of workers at a younger age (Seehuus et al., 2006; Amdam et al., 2007; Nelson et al., 2007).

Furthermore, the inhibitory effect of these molecules has been shown to go both ways. High levels of JH will inhibit the synthesis of Vg, maintaining bees in the foraging stage. This being said, the relationship between JH and Vg has been proven to be more complicated than this as low levels of JH do not inhibit Vg production and curiously, the presence of JH is necessary for Vg production

(Fluri et al., 1977; Pinto et al., 2000; Amdam and Omholt, 2002; 2003; Amdam et al., 2003; Guidugli et al., 2005).

The effects of nurse nutrient stores on the lifespan of worker bees is believed to be (at least partly) induced by the general impacts of brood rearing (Amdam and Omholt, 2002; Amdam et al., 2009; Smedal et al., 2009). Nursing has been shown to substantially affect Vg levels in worker bees and an inverse relationship between brood load and Vg levels has been illustrated (Amdam et al., 2009; Van der Steen et al., 2015). Additionally, when colonies are artificially made broodless, Vg levels increase (Amdam et al., 2009; Smedal et al., 2009; Van der Steen et al., 2015; Eyer et al., 2017). Thus, when the nurse load is high, Vg nurse levels will drop, giving rise to JH, accelerating the transition to the forager stage and decreasing the lifespan of worker bees (Amdam and Omholt, 2002; Amdam et al., 2009). This inverse relationship between Vg levels and nurse load is a logical one as intrinsic Vg is utilized as a precursor molecule in the production of jelly by nurse bees (Amdam and Omholt, 2002; Amdam et al., 2003; Ricigliano et al., 2018). Additionally, pheromones produced by brood have been shown to impede the Vg storage capacity of older bees offering an added explanation for the nutrient reducing effects of brood rearing (Smedal et al., 2009).

This being said, general starvation of a hive will cause premature onset of foraging, illustrating the accelerated nurse-to-forager transition mechanism to lie within the overall amount of nutrient stores of the bees, and not solely the intensity of brood rearing (Schulz et al., 1998). Moreover, as starvation has been shown to causes an increase in Op in invertebrates, it is likely the same to be true in bees and thus worker bee nutrient stores to affect the age of onset of foraging through this particular pathway (Toth and Robinson, 2005).

1.2.2. Regulation based on brood microclimate

It can be argued that the longevity reducing effect of the brood load is (additionally) explained by an alternative mechanism involving what is called the brood microclimate (Mattila et al., 2001; Mattila and Otis, 2007; Döke et al., 2015). Honey bee larva require a specific microclimate environment for optimal development and growth and worker bees are, consequently, very precise in maintaining these conditions (Nürenberger et al., 2018). As a result, the brood core within a hive has a constant temperature of 35 °C and 1.5% CO_2 (Mattila et al., 2001; Döke et al., 2015). Immersion within this specific microclimate triggers JH to rise within worker bees. Thus, when brood rearing is high, the brood core grows and an elevated number of worker bees will find themselves within this microclimate, causing their JH levels to rise, possibly accelerating their switch to forager bees. Regardless, the effects of this mechanism have so far been poorly investigated and its workings mostly used as a possible explanation regarding the contrary; the increased longevity of workers emerging at times of decreased brood load (Mattila et al., 2001; Mattila and Otis, 2007; Döke et al., 2015).

1.2.3. Regulation based on colony age demography

Finally, the age at which bees start foraging is controlled by a pheromone-mediated system of social inhibition and modulation responding to changes in colony age demography. The workings of this system are intended to assure an adequate proportion of each worker bee caste within a colony. So far, three forms of social modulation have been recognized within honey bee colonies; worker-worker interaction, brood pheromone, and queen pheromone (Huang and Robinson, 1992; 1996; Pankiw et al., 1998; Le Conte et al., 2001; Leoncini et al., 2004; Toth et al., 2005; Russell et al., 2013; Eyer et al., 2017).

Worker-worker interaction is mostly defined by the inhibitory effect of foragers on young bees. Adult honey bees produce a pheromone called ethyl oleate. The production of this pheromone increases with age and is substantially higher in foragers. Subsequently, ethyl oleate is passed from foragers to the in-hive bee population through trophallaxis together with collected nectar (Leoncini et al., 2004). When a hive consists of an adequate amount of foragers, the strength of their pheromone signal delays the transition of young bees to the forager stage. On the other hand, when a hive lacks in foragers, ethyl oleate levels will drop, accelerating this transition and thus insuring a stable ratio between in-hive and forager bees (Huang and Robinson 1992; 1996; Leoncini et al., 2004). Additionally, recent research by Eyer et al. (2017) has demonstrated that the presence of young adult bees affects worker lifespans as well. Although no underlying mechanism has yet been identified, a reduction in longevity was recorded in the presence of young bees and the absence of brood.

Similarly, brood also influences the age of onset of foraging in worker bees. Brood pheromone, a blend of fatty-acid esters (10 components) produced by brood has been shown to delay the transition of young bees to the foraging stage as prominent exposure to this pheromone indicates a high need for nursing (Le Conte et al., 2001). Contrary, Maisonnasse et al. (2010) determined E-β-ocimene, a volatile pheromone produced by brood, to accelerate the maturation of nurse bees, insuring sufficient pollen supplies for brood rearing.

Finally, honey bee queen mandibular pheromone has been shown to delay the transition to out-hive bees, for less obvious reasons (Pankiw et al., 1998).

Accelerated and delayed transitions to the foraging stage due to colony age demography are associated with premature or delayed rises in JH titers respectively (Huang and Robinson, 1992; 1995; 1996; Pankiw et al., 1998; Le Conte et al., 2001) as well as accompanied by changes in antennal lobe Op levels (Schulz et al., 2002a).

2. The winter bee

In northern regions, bees present from October until March are categorized as winter bees (long-lived bees), whereas bees found during the rest of the year are labelled as summer bees (short-lived bees). September and April can be considered transition months as most likely a balanced number of both populations of bees is present at those times (Mattila and Otis, 2007; Döke et al., 2015; Kunc et al., 2019). This being said, as the timescale regarding seasonal change will vary based on geographical location, this distinction is somewhat theoretical (Fukuda and Sekiguchi, 1966; Ricigliano et al., 2018). Additionally, formation of “winter bees” has also been shown to occur within warmer climates where impaired environmental conditions leading to the depletion of nutrient supplies result in a decrease in brood rearing and an increase in Vg levels among worker bees (Amdam et al., 2005b; Döke et al., 2015; Ricigliano et al., 2018).

Overall, the formation of winter bees is an adaptive mechanism allowing honey bee colonies to bridge unfavourable variations in temperature and food availability (Mattila et al., 2001; Mattila and Otis, 2007; Döke et al., 2015; Nürenberger et al., 2018; Ricigliano et al., 2018). During these dire conditions, winter bees will remain within the hive tending to colony survival (Seehuus et al., 2007). First of all, long-lived winter bees are tasked with in-hive thermoregulation, accomplished by the formation of a thermoregulating cluster. Herein, heat is generated by the bees at the centre of the cluster intensively vibrating their flight muscles. By doing so, winter bees are kept from experiencing a lethal drop in temperature, allowing the colony to outlast the cold winter months (Stabentheiner et al., 2003; Döke et al., 2015; Nürenberger et al., 2018). During this time, bees will use honey, stockpiled during summer, as their only energy source (Amdam et al., 2005b; Döke et al., 2015; Kunc et al., 2019). Second of all, winter bees store large amounts of lipids and protein within their bodies, effectively transforming into a “nutrient storage caste” (Amdam and Omholt, 2002; Amdam et al., 2003; 2005b; Ricigliano et al., 2018; Kunc et al., 2019). These stored nutrients are conserved throughout winter and subsequently utilized to reinitiate brood rearing when the return of favourable environmental conditions is anticipated (Münch et al., 2013; Ricigliano et al., 2018). Nutrient conservation is achieved by restriction of overall and metabolic activity (Van Nerum and Buelens, 1997; Amdam and Omholt, 2002; Döke et al., 2015). The fact that winter bees all die at approximately the same time, regardless of the time of eclosion, illustrates their specific function regarding the initiation of brood rearing for the next cycle (Mattila et al., 2001). In general, the winter bee population will vanish towards the end of spring once the new cycle is well on the way (Fukuda and Sekiguchi, 1966; schulz et al., 1998; Mattila and Otis, 2007).

Honey bee workers are able to transform into the winter physiological state under various conditions. Modelling of this transition has revealed that not only bees deprived of any nursing or foraging, but also bees experiencing a normal nurse load and even bees performing foraging tasks for a short period of time to be able to undergo the switch to *diutinus* bees as long as they have access to ample nutritive recourses (Amdam and Omholt, 2002).

2.1. Vitellogenin and its central role in winter bee physiology

Vitellogenin plays a central role in winter bee physiology and is fundamental for instigating the various features typically associated with *diutinus* bees, including: a) high hemolymph and fat body lipid and protein contents; b) hypertrophied HPGs, and c) longevity (Amdam and Omholt, 2002; Amdam et al., 2003; 2004; 2005b; Seehuus et al., 2006; Münch et al., 2013; Kunc et al., 2019).

Transformation into the “winter bee nutrient storage caste” requires the accumulation of large amounts of lipids and protein. Conveniently, honey bees can achieve this by stockpiling one single molecule: Vg, which is produced and stored by the fat body (Amdam and Omholt, 2002; Amdam et al., 2005b; Toth et al., 2005; Ricigliano et al., 2018; Kunc et al., 2019). The fat body, the central regulatory tissue for metabolism and nutrient storage within honey bees is comparable with the liver (combined with the white adipose tissue) in vertebrates (Seehuus et al., 2007; Münch et al., 2013; Döke et al., 2015; Kunc et al., 2019). In bees, this organ is located in the abdomen and to a lesser extent in the head (Seehuus et al., 2007; Münch et al., 2013).

The ability of nurse bees to conserve and build up their nutrient stores (Vg) for the coming winter months has been hypothesized to be the result of the gross decrease in brood rearing observed in honey bee colonies during fall (Amdam and Omholt, 2002; Amdam et al., 2005b; 2009; Mattila and Otis, 2007; Smedal et al., 2009; Kunc et al., 2019). The protein content of the fat body of honey bee workers increases substantially during this period (Amdam and Omholt, 2002). Furthermore, the correlation between brood rearing and worker bee Vg titers is illustrated by the fact that overall Vg levels within a hive follow a seasonal pattern antagonistic to that of brood rearing (Van der Steen et al., 2015).

Overall, even though brood food and HPGs only contain a minimal amount of Vg, production of jelly for brood rearing is believed to be a prominent pathway depleting nurse Vg stores under normal circumstances (Amdam and Omholt, 2002; Amdam et al., 2003; 2005b; 2009; Seehuus et al., 2007). Most likely, HPGs have an efficient processing apparatus for Vg, producing products that will eventually make up brood food. Incidentally, Vg is mobilized from the fat body into the haemolymph where it is shunted to the HPGs. Next, Vg is taken in and catabolized into lipids and amino acids, which are then utilized to produce proteinaceous jelly (Amdam and Omholt, 2002; Amdam et al., 2003). Although this mechanism has not yet been fully proven, strong evidence exists supporting this hypothesis (Amdam et al., 2003; Seehuus et al., 2007). Additionally, existence of such a metabolic pathway would offer a reliable explanation for the high level of Vg synthesis, an egg-yolk precursor protein commonly produced by oviparous female insects, in otherwise functionally sterile female workers (Amdam and Omholt, 2002; Amdam et al., 2003; 2005b; Seehuus et al., 2007).

Hypertrophied HPGs most likely results from the same mechanism described above (Mattila et al., 2001; Amdam and Omholt, 2002; Amdam et al., 2003; Mattila and Otis, 2007; Seehuus et al., 2007; Döke et al., 2015; Kunc et al., 2019). This is illustrated by the fact that summer worker bees with restricted access to brood will show such hypertrophied glands. As well, nursing reduces the overall size and protein content of HPGs (Amdam and Omholt, 2002). When brood rearing declines, so does the need for jelly and thus, Vg builds up in the fat body and haemolymph of young worker bees (Amdam et al., 2005b; Mattila and Otis, 2007; Smedal et al., 2009; Kunc et al., 2019). Presumably, Vg will still be taken up by the HPGs (to the extent possible) and its metabolites accumulated in their acini, substantially increasing the size of these glands. Therefore, it is plausible that, in times of decreased brood rearing, HPGs serve a storage organ similar to the fat body. This hypothesis is backed by the exceedingly elevated protein content of the HPGs of winter bees (Amdam and Omholt, 2002). Additionally, the presence of prevalent HPGs is associated with low JH titers, typical for the winter bee (and nursing) state (Huang and Robinson, 1995; 1996; Amdam et al., 2005a; Mattila and Otis, 2007; Döke et al., 2015; Kunc et al., 2019). As JH has been shown to cause HPG atrophy (Fluri et al., 1977), consistent inferior JH levels might enable the reverse response.

The accumulation of large amounts of Vg is strongly correlated with the longevity of winter bees and therefore considered of great importance for overwintering. This longevity extending effects of Vg can be accredited to two separate mechanisms involving this molecule (Amdam and Omholt, 2002; Amdam et al., 2004; 2005b; 2009; Seehuus et al., 2006; Smedal et al., 2009; Ricigliano et al., 2018; Kunc et al., 2019).

Vitellogenin is a well-known zinc (Zn) carrier and haemolymph Zn titers show a strong correlation to Vg contents in bees (Amdam and Omholt, 2002; Amdam et al., 2004). As Zn is known to protect cells against oxidative stress, haemolymph Vg titers have been hypothesized as a major component protecting winter bees from oxidative damage and thus having lifespan-extending properties (Amdam and Omholt, 2002; Seehuus et al., 2006). Vitellogenin has also been shown to have anti-oxidative properties in other organisms (Amdam and Omholt, 2002) and is thought to be at least partly responsible for instigating the lengthy lifespan of queen bees in this manner (Corona et al., 2007).

This mechanism has further been demonstrated by Seehuus et al. (2006) who showed Vg to preferentially be oxidized (scavenging free radicals), acting as a protective agent against oxidizing components. Artificial reduction of Vg in this study lead to an increase susceptibility to oxidative stress-inducing molecules. As a clear correlation between lifespan and oxidative stress resistance has been pointed out before, this process offers a reliable insight on the longevity extending properties of Vg (Seehuus et al., 2006).

Next, an association between Vg titers and immune function has been demonstrated in honey bees. In this regard, Vg enhances innate immunity, possibly supporting increased longevity in winter bees. This association was first reported in forager bees which show a decreased cellular immunity (reduced haemocytes or immunocytes) coupled to faded Vg stores and increased JH levels. The impaired immunity in forager bees is the result of decreased haemolymph Zn contents as, besides having anti-oxidative function, this element is required for normal immunocyte function. Depletion of Vg and, consequently Zn, leads to apoptosis in immune cells among others. Cellular immunity is central in processes like phagocytosis, encapsulation, nodulation, antibacterial activity and wound healing and thus paramount for the overall immune function of bees (Amdam et al., 2004, 2005a). Additionally, increased JH levels has also been accredited to cause death of immune cells in forager bees, offering a possible added explanation for the association between reduced Vg and immune function as these two molecules are strongly interlinked (Amdam et al., 2005a).

Then again, available literature regarding the cellular immunity of winter bees is contradictory (Amdam and Omholt, 2003; Amdam et al., 2004; 2005a; Kunc et al., 2019). Overall, it is accepted that cellular immunity is downregulated in bees during winter. Moreover, research on potential biomarkers for *diutinus* bees revealed no significant difference in haemocyte count between summer and winter populations (Kunc et al., 2019), contrary to previous observations (Amdam et al., 2005a). Overall, winter bees will maintain these typical properties throughout winter, until resumption of brood rearing (Huang and Robinson, 1995; Mattila et al., 2001; Mattila and Otis, 2007; Döke et al., 2015; Kunc et al., 2019).

2.2. The winter bee transition

All in all, transition into the winter bee state can be explained by extension of the various mechanisms determining worker bee life span plasticity explained above (Amdam and Omholt, 2002; Amdam et al., 2009; Smedal et al., 2009; Döke et al., 2015; Eyer et al., 2017). Hitherto, varying triggers have been argued to be responsible for this physiological transition, including social and environmental cues. A schematic representation of the in- and out-hive factors believed to influence the summer to winter bee transition is given in Figure 3.

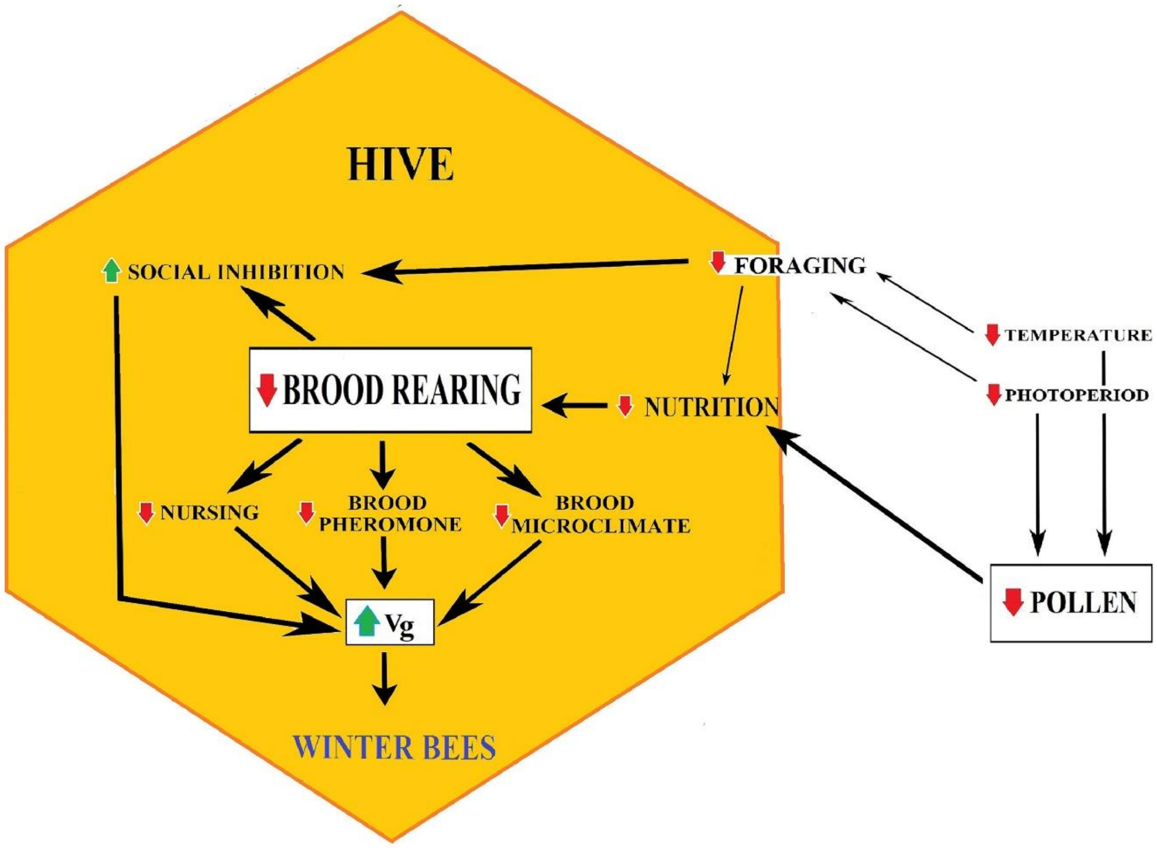


Figure 3. Schematic representation of the in- and out-hive factors influencing the summer to winter bee transition. (VG= Vitellogenin). The gravity of each factor is expressed by the thickness of the arrows.

Although the workings of this transition have not yet been unravelled to the full extent, substantial evidence has been produced indicating the arrival of winter bees to be strongly correlated to one factor in particular, namely the overall decrease in brood rearing activity observed in fall (Mattila et al., 2001; Amdam and Omholt, 2002; Mattila and Otis, 2007; Amdam et al., 2009; Smedal et al., 2009; Münch et al., 2013; Döke et al., 2015). The lack of brood alone has been shown to be able to cause colonies to transition to their winter state, regardless of other (possible) triggers like photoperiod, temperature or availability of pollen (Amdam et al., 2005b; Mattila and Otis, 2007; Döke et al., 2015). Likewise, research has shown the lifespan of newly emerging worker bees to be inversely correlated to the amount of brood being reared at the time of eclosion (Mattila et al., 2001; Mattila and Otis, 2007; Amdam et al., 2009; Russell et al., 2013) and if brood rearing in fall is artificially maintained at a high intensity, short-lived worker bees are produced (Mattila and Otis, 2007; Eyer et al., 2017). Regardless, it is still unclear how a decrease in brood load triggers the winter bee transition (Döke et al., 2015; Nürenberger et al., 2018).

It is likely the effect of brood rearing on nurse bee nutrient stores to be a key factor in the winter bee transition. As explained extensively in previous paragraphs, a decrease in colony brood rearing results in a steep decrease in nutritional pressure for nurse bees, enabling young bees to retain their nutritional stores. Consequently, elevated Vg levels will prevent JH titers to rise, maintaining bees in the nursing state and extending their longevity. Further, absence of the suppression of JH prevents Vg synthesis to be shut down, allowing these bees to further build up their nutrient stores, effectively transforming into their winter state (Fluri et al., 1977; Amdam and Omholt, 2002; Amdam et al., 2003; 2005b; 2009; Mattila and Otis, 2007; Smedal et al., 2009; Kunc et al., 2019). In addition, reduction in brood load concurs with a reduction in brood pheromone, likely allowing worker bees to retain their Vg storage capacity for a prolonged period of time. This mechanism correlates well with the established knowledge regarding the winter bee transition as prolonged elevated Vg levels would allow continuous JH suppression (Smedal et al., 2009). Finally, the effects mediated by a reduced brood microclimate might also play a part by keeping the JH levels of newly emerging bees in check (Mattila et al., 2001; Mattila and Otis, 2007; Döke et al., 2015). In this regard, as the brood core diminishes in fall, a sizable amount of worker bees will find themselves outside of this microclimate, preventing JH to rise, delaying their transition to the foraging stage, resulting in increased longevity. Moving forward in the winter months, this effect will continue to increase as brood rearing decreases further, giving rise to the winter bee population (Mattila et al., 2001; Mattila and Otis, 2007).

This hormonal mechanism, determined by the brood load and brood microclimate, provides viable explanations for the gradual, seasonal appearance of long-lived winter bees within a hive (Mattila et al., 2001; Mattila and Otis, 2007; Amdam et al., 2009). Moreover, these hypotheses are well fitted within the general knowledge regarding the hive dynamics of JH. Hive JH titers decrease around October-November, dip in January and subsequently increase steeply in February-March in correspondence with general brood rearing patterns (Huang and Robinson, 1995; Döke et al., 2015).

Next, it is noteworthy to mention that the seasonality of the brood rearing pattern of honey bees is no coincidence, and by extension, factors regulating this pattern can be considered key triggers in the determination of the appearance of long-lived winter bees (Mattila et al., 2001; Mattila and Otis, 2007; Russell et al., 2013; Döke et al., 2015; Nürenberger et al., 2018). In this way, the appearance of winter bees is strongly correlated to naturally plummeting pollen supplies in fall (Mattila and Otis, 2007; Russell et al., 2013). Predominantly, honey bee colonies do not keep large stores of pollen (contrary to honey) (Russell et al., 2013; Wood et al., 2018) which, is the main nutrient supply necessary for brood rearing (Pankiw et al., 1998; Keller et al., 2005; Mattila and Otis, 2007; Döke et al., 2015; Wood et al., 2018). Therefore, at any given time, the brood rearing capacity of a colony is directly related to the pollen availability. When pollen is plentiful, brood rearing in bees is high. Contrarily, when pollen supplies are scarce, brood rearing is substantially downregulated. For this reason, honey bees exhibit an annual brood rearing cycle following seasonal pollen availability (Keller et al., 2005; Mattila and Otis, 2007; Russell et al., 2013; Döke et al., 2015). Furthermore, research by Mattila and Otis (2007) showed that prolonged pollen availability delays the emergence of winter bees within a hive without affecting the total winter-bee-population size, providing evidence this trigger to be responsible for the timing of winter bee development. Additionally, artificial reduction of pollen availability will accelerate transition of a colony into the winter state (Mattila and Otis, 2007). Thus, the seasonal effect of pollen availability on the brood rearing capacity of a colony can be concluded to effectuates the bimodal annual longevity distribution seen in honey bees and to be a primary trigger instigating the summer to winter bee transition (Mattila and Otis, 2007; Russell et al., 2013; Döke et al., 2015; Yamada et al., 2019).

Moving on, previous research efforts have investigated the effects of climatological factors on the arrival of winter bees as well. Hereby, focus has been put on the decrease in both ambient temperature and photoperiod as these environmental changes typically indicate the arrival of winter. In this regard, these external factors were identified to only be partially involved in the matter, if at all (Mattila et al., 2001; Mattila and Otis, 2007; Döke et al., 2015). Experimental studies have not been able to induce declines in brood rearing activities by artificially manipulating temperature and photoperiod (within naturally occurring parameters) either separate or simultaneously (Mattila et al., 2001; Mattila and Otis, 2007). Thus, these environmental cues alone could be dismissed as primary triggers for the physiological development of winter bees (Mattila et al., 2001; Mattila and Otis, 2007). Most likely, the effect of these environmental cues is rather an indirect one, influencing the pollen supply for example, which in turn affects brood rearing (Mattila and Otis, 2007).

Alternatively, decreasing ambient temperatures and photoperiod were hypothesized to possibly influence the arrival of winter bees through the restriction of foraging activities of older bees (Döke et al., 2015). Firstly, limiting the foraging capacity of a hive might lead to similar effects as overall decreasing pollen supplies regardless of the presence of floral resources (Mattila and Otis, 2007; Döke et al., 2015). Secondly, restricted out-hive activity of foragers means an increase in time spent in-hive, influencing the pheromone balance of a colony. Consequently, young bees will be subjected to higher amounts of negative feedback through the workings of ethyl oleate (Huang and Robinson 1992; 1995; 1996; Leoncini et al., 2004; Döke et al., 2015). This increase in social inhibition, just like the effects of the brood microclimate and Vg, would inhibit JH titers to rise in newly emerging bees, allowing these to instigate transition to the winter bee state (Huang and Robinson, 1992; 1995; 1996; Mattila et al., 2001; Döke et al., 2015). This hypothesis is supported by the findings that the transition of young bees to the foraging stage is delayed by adverse weather conditions, an effect accredited to increased worker-worker interactions as foragers are impeded from leaving the hive (Schulz et al., 1998).

Furthermore, the overall decreasing amount of brood reared in fall will also cause changes in colony demography. Older bees will make out a larger proportion of the hive, increasing the suppression instigated by the workings of ethyl oleate, inhibiting maturation of newly emerging bees and increasing their longevity (Huang and Robinson, 1995; Mattila et al., 2001; Leoncini et al., 2004).

The two-tiered mechanism described above as responsible for the transition between summer and winter bees is a very elegant one. As the shift to the winter bee state is governed by the seasonal decrease in pollen availability rather than based on environmental cues, this transition is guaranteed even if the timing of changing environmental factors is accelerated or delayed from year to year. In this way, honey bee colonies can directly regulate their overall activity, regardless of varying climatological factors (Mattila and Otis, 2007).

2.3. The summer bee transition

Compared to the winter bee transition, transition back into the summer state has been investigated to much less extent (Döke et al., 2015; Nürenberger et al., 2018). In this case it can be argued that the return of brood rearing activities will trigger a decline in Vg levels in winter bees, allowing for JH titers to finally rise and take over (Huang and Robinson, 1995; Amdam and Omholt, 2002; Amdam et al., 2003; 2005b; 2009; Mattila and Otis, 2007; Döke et al., 2015; Kunc et al., 2019). As brood rearing is initiated in mid-winter, no floral recourses are available for winter bees to maintain their nutrient stores (Huang and Robinson, 1995; Mattila and Otis, 2007; Puškadija et al., 2017). Additionally, cold ambient temperatures will most likely inhibit bees from venturing outside the hive in any case (Huang and Robinson, 1995; Amdam and Omholt, 2002). Thus, even though the brood load in mid-winter is most likely low and not causing extensive nutritional pressure for the winter bees, inability to replenish nutrient stores will cause sufficient decrease in Vg titers to allow eventual transition back into a summer type physiology.

Next, the inverse effect of that seen during the winter bee transition regarding the brood microclimate can also apply. As brood rearing is upregulated, the brood microclimate within a hive will grow, allowing for JH titers to rise in bees finding themselves within the brood core, initiating the transition back to the summer physiological state (Mattila et al., 2001; Mattila and Otis, 2007; Döke et al., 2015).

Once JH worker-levels have increased sufficiently, bees will start venturing outside the hive again in search of pollen (Huang and Robinson 1992; 1995). This influx of pollen into the hive will in turn promote brood rearing, boosting the transition back to summer bees (Huang and Robinson, 1995;

Mattila and Otis, 2007; Russell et al., 2013; Döke et al., 2015; Yamada et al., 2019). Finally, as the new generation of bees grows (and the winter population dies off), honey bee colonies will show an abundance of young bees, with low ethyl oleate levels, promoting an accelerated transition to the forager stage, allowing hives to return to their summer composition (Huang and Robinson 1992; 1995; 1996; Leoncini et al., 2004; Döke et al., 2015). Furthermore, an increasing amount of brood will lead to the return of the "normal" mixture of brood pheromones which will further stabilize the demographic distribution of honey bee colonies seen during the rest of the year (Maisonnasse et al., 2010; Döke et al., 2015; Nürenberger et al., 2018).

This being said, very little is known regarding the timing of initiation of brood rearing in honey bee winter colonies and what triggers are used to predict the approach of favourable reproductive circumstances (Döke et al., 2015; Nürenberger et al., 2018). The effects of changing environmental factors have somewhat been investigated in this regard and were shown to have some influence. Regardless, extensive further research is needed on the matter (Döke et al., 2015; Nürenberger et al., 2018). Overall it is accepted that changes in ambient temperature as well as changes in photoperiod duration affect brood rearing in winter bees (Nürenberger et al., 2018). Additionally, Nürenberger et al. (2018) were able to demonstrate that an increase in temperature causes earlier onset of brood rearing compared to continuous cold weather. The experiments during this research also indicated that changes in photoperiod alone do not have a causal effect on brood rearing in winter honey bee colonies. However, the results of this study suggest that an interplay between these two factors might be necessary to trigger brood rearing in winter and thus photoperiod only to have an effect in combination with increasing ambient temperatures. Finally, Nürenberger et al. (2018) concluded that, based on the intensity of the response to changes in ambient temperature and photoperiod, most likely additional triggers are utilized by colonies to determine optimal timing in brood rearing onset. Moreover, it is improbable that ambient temperature serves as a dominant trigger for onset of brood rearing as this practice is initiated during mid-winter, the coldest time of the year (Döke et al., 2015).

In addition to the effects of environmental cues, it would appear that bees have an internal clock affecting the initiation of brood rearing, as an increase in brood rearing activity was found over time, regardless of temperature and/or photoperiod (Nürenberger et al., 2018). This new finding is not surprising given the well-established time keeping ability and behavioural rhythmicity of honey bees. In this regard, a variety of honey bee activities, including regulation of activities on a group scale, have been known to operate according to a circadian clock or "zeitgeber" (Moore, 2001). Furthermore, environmental factors like temperature and photoperiod were shown to influence zeitgeber function in honey bees (Moore, 2001), offering a potential pathway behind the effect of these factors in seasonal honey bee transitions.

Finally, it is noteworthy to mention that pollen availability can be dismissed as a primary cue for the transition back to summer physiological state as brood rearing is initiated in winter when floral recourses are still deficient (Mattila and Otis, 2007).

3. Conclusion

Overall, previous research efforts have led to a detailed description of the physiological features of both seasonal types of worker bees and the functionality of the various molecules central to their biology. The unique adaptations of JH and Vg in honey bees has not only allowed these insects to develop an effective social system for the division of labour but also to adjust to climatological extremes (at least in insect terms) and thrive in the Northern parts of the world.

Although our understanding of many of the pathways involving these molecules is more than adequate, some aspects, like the mechanism behind brood food production and the immune function of Vg, warrant further attention.

Current knowledge regarding the seasonality of honey bee colonies has enabled us to paint an elegant picture describing a multi-tiered system responding to in- and out-hive cues with variable sensitivity. Considerable progress has been made in regard to the summer to winter bee transition especially. Sufficient evidence has been produces to confidently point to the overall decrease in brood rearing activity and the plummeting pollen supply in fall as the primary triggers behind the appearance of winter bees. Moreover, the current understanding of honey bee physiology has led to the most interesting insight in how these social insects have “transformed” existing regulatory pathways for short term labour distribution into a bimodal, biannual worker caste system enabling honey bees to cope with the seasonal loss of favourable conditions.

This being said, the roles and gravity of secondary cues, like ambient temperature and photoperiod, remain less obvious and need to be investigated more extensively in order to correctly be placed in the overall picture. On the other hand, the effects of secondary triggers are, most likely, heavily intertwined and/or exhibit influence on multiple levels, making their roles harder to quantify.

Additionally, quantification of the gravity of the different mechanisms driving the division of labour in worker bees within seasonal transitions is lacking. Overall, the effects of nurse bee nutrient stores seem to be central within this phenomenon, which concurs with the main hypotheses behind the seasonal transition of honey bee colonies. Regulation based on colony demography appears to be crucial for the maintenance of proper labour distribution in summer colonies, but lesser in regards to colony transitions. Lastly, very little research has been conducted surrounding the effects of brood microclimate on both fronts and thus warrants further attention.

Finally, the workings of the transition back from winter to the summer bee state has been investigated to much less extent and still represents a significant research gap. Although multiple aspects of this specific transition can be explained by the antagonistic function of the same mechanisms used in the summer to winter bee transition, this cannot be said for its primary trigger and thus, so far, remains unknown. Future research efforts should therefore, besides on clarifying the secondary triggers for the summer to winter bee transition and the importance of the different mechanisms driving the worker bee division of labour, focus on the unravelling of the principal cues behind the return of a colony to its classical summer state.

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DECLARATION OF COMPETING INTEREST

Authors declare that no conflict of interest exists.

CHAPTER 3

Preliminary evaluation of selected morphological metrics for honey bee nutrition and health assessment

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Highlights

- Research and monitoring of *Apis mellifera* through markers of honey bee health is of increasing importance in the context of global colony losses.
- Markers representative of the nutritional status of individual bees show great promise.
- A preliminary assessment of robust size measurements of individual workers as possible markers of honey bee health and nutrition is presented.
- Through correlation analysis and monitoring of selected metrics over a 7 month period, abdominal length, head width, and total body length measurements were identified as candidate markers of honey bee nutrition and health.

Abstract

The unsustainable degree of winter honey bee colony losses in present days has raised considerable concern. While causal factors behind this phenomenon are not fully understood, colony losses have been accredited to a multitude of simultaneous and interacting stressors. Further research is required to better understand the effects of such stressors, which is best achieved through the monitoring of honey bee health using a biomarker approach. The identification of novel easy-to-use biomarkers representative of honey bee health and nutrition would prove beneficial, especially across temporal patterns. For this reason, this research aims to evaluate selected metrics of individual size (head width, thoracal width and length, abdominal width and length, and total body length) as possible markers for honey bee health. As a proof of concept, selected morphological parameters were monitored in forager bees through monthly sampling over the transition period across autumn-winter-spring and compared with established markers of honey bee health and nutrition. Results showed significant size variation of individual bees over the sampling months and correlation analysis revealed abdominal and total body length to be potentially suited markers for the nutritional status of foragers. Analysis of variance highlighted differences in the nutritional status of forager bees in December specifically and abdominal length to be a suitable candidate marker of honey bee nutrition across temporal patterns. While head width was not shown to be strongly correlated to nutrition in foragers, this metric could prove to be a useful seasonal marker. Findings of this research illustrate the potential of robust measurements of individual size as markers for the nutritional and health status of honey bees across temporal patters, although further research is required to validate these metrics.

KEYWORDS

Honey bees, Forager, Morphometry, Biomarker, Nutrition

1. Introduction

The honey bee (*Apis mellifera* Linnaeus, 1758) is a highly significant semi-domesticated insect valued for its various products and services. The global demand for honey, the most prized of the bee products, is ever-increasing (Gupta et al., 2014; Phiri et al., 2022) with a reported worldwide production of 1.77 million tonnes in 2021, worth USD 8.58 billion (FAO, 2020; Grand View Research, 2021). Nevertheless, the value of the ecosystem services provided by this animal in terms of pollination of wild plants and agricultural crops is even more substantial (Aizen et al., 2009; De La Rúa et al., 2009; vanEngelsdorp and Meixner, 2010; Leonhardt et al., 2013; Klein et al., 2018). As an estimated 80% of wild flora (De La Rúa et al., 2009) and roughly 70% of the world's leading food crops are believed to benefit from honey bee pollination (Klein et al., 2007; Klatt et al., 2014; Stein et al., 2017; Nath et al., 2023), this eusocial insect plays a key role in ecosystem and biodiversity preservation, as well as agricultural sustainability and food security in a modern climate (Espregueira et al., 2020; Nath et al., 2023).

Since the mid-20th century, severe declines in managed honey bee populations have been reported in Europe and North America raising considerable concern (Potts et al., 2010; 2016; vanEngelsdorp and Meixner, 2010; Goulson et al., 2015; Aizen et al., 2022). Additionally, an unsustainable degree of global honey bee colony losses is seen in present days, especially in winter (Ellis et al., 2010; Neumann and Carreck, 2010; Jacques et al., 2017; López-Uribe et al., 2020; Halvorson et al., 2021; Insolia et al., 2022). Latest reports show an average loss rate of 16.4% across Europe, with some countries reporting >35% colony mortality during the winter of 2019-2020 (Gray et al., 2022). In the United States, colony losses averaged a staggering 39.0% in 2021-2022, with losses of 23.8% and 24.2% in summer and winter respectively (Aurell et al., 2022).

Honey bee colony losses have been accredited to the effects of a multitude of factors, including (but not limited to) pests and diseases, pesticide use, environmental contamination, climate change, agricultural intensification, habitat loss, and malnutrition (De La Rúa et al., 2009; Neumann and Carreck, 2010; Goulson et al., 2015; Potts et al., 2016; Steinhauer et al., 2018; Kim et al., 2022). However, identifying clear causal factors behind colony losses has proven difficult likely due to the simultaneous influence of multiple stressors with varying modes and degrees of interaction (Goulson et al., 2015; Steinhauer et al., 2018).

Research efforts to further identify, evaluate, and quantify factors influencing colony losses are of increasing importance in order to prevent further large-scale losses and to guarantee the sustainability of apiculture and its services (Le Conte and Navajas, 2008; Jacques et al., 2017; Switanek et al., 2017; Nath et al., 2023). In this regard, the monitoring of colonies through biomarkers representative of honey bee health represents a very useful and unbiased approach. A significant advantage would be the (real-time) monitoring of colonies, allowing for early implementation of measures preventing unnecessary losses (López-Uribe et al., 2020). Nevertheless, further efforts are required to identify useful biomarkers of honey bee health, especially across temporal (e.g. seasonal) and spatial patterns (Münch et al., 2013; Smart et al., 2016; Alaux et al., 2017; 2018; Ricigliano et al., 2018; 2019; Kunc et al., 2019; López-Uribe et al., 2020).

While the quantification of honey bee health can be achieved through a variety of metrics (López-Uribe et al., 2020), measurement of nutritional markers of individual bees has emerged as a method of particular interest (Brodschneider and Crailsheim, 2010; Smart et al., 2016; López-Uribe et al., 2020). Overall, these markers reflect colony demography as an indicator of colony health (López-Uribe et al., 2020), and include biochemical quantifications (e.g. lipid and protein concentration), fat body and hypopharyngeal gland (HPG) size estimation, vitellogenin (Vg) levels, and body size measurements among others. Additionally, the majority of these metrics have been shown to be useful indicators in the context of winter colony losses as they reflect the presence and quantity of long-lived winter bees, a marker of particular importance for overwintering success (Wilson-Rich et al., 2008; Alaux et al., 2010; 2017; 2018; Di Pasquale et al., 2016; Smart et al., 2016; Kunc et al., 2019; Ricigliano et al., 2018; 2019; Watkins de Jong et al., 2019; López-Uribe et al., 2020; Koubová et al., 2021).

This research aims to evaluate selected easy-to-use measurements of individual size as possible markers for honey bee health and nutrition. As a proof of concept, selected morphological parameters of honey bee workers were monitored through monthly sampling over the transition period across autumn-winter-spring and compared with established markers of honey bee health. This scheme was explicitly chosen as (in temperate zones of the northern hemisphere) worker bees take on two distinct forms within an annual cycle (summer *Vs*. winter bees) with physiological differences heavily dictated by their nutritional status (Knoll et al., 2020). Specifically, winter bees store large amounts of fat and protein within their bodies, resulting in (extremely) elevated Vg levels and the enlargement of their fat bodies and HPGs (Mattila et al., 2001; Amdam and Omholt, 2002; Amdam et al., 2003; 2005; Mattila and Otis, 2007; Döke et al., 2015; Ricigliano et al., 2018; Kunc et al., 2019). Authors hypothesized this transformation into a “nutrient storage caste” would result in notable morphological alterations. Detection of winter bees using selected morphological parameters (and confirmed through known markers) would serve as a first step to recognise general size variations among worker bees as a reflection of their nutritional status and to be a potentially useful marker of honey bee health across temporal patterns.

2. Materials and methods

2.1. Study area, experimental bees, and sample collection

Samples for this research were collected from an experimental apiary in the province of Sassari (40° 39’ 9”36 N; 08° 22’ 52”32 E), Sardinia (Italy), from November 2019 until May 2020. Sardinia, located directly West of mainland Italy, is the second largest island in the Mediterranean Sea and is characterized by hot and dry summers with mild and wet winters; southern temperate Mediterranean climate.

Samples were collected from a single hive of Italian honey bees (*Apis mellifera ligustica* Spinola, 1806) to minimize genetic variance. The hive in question was selected by the professional beekeeper in charge of the apiary based on overall health. The experimental colony was maintained through standardized practices and was regularly inspected insuring adequate health over the course of the study. No clinical signs of disease were noted. Treatment against the ectoparasitic mite *Varroa destructor* was applied in November using oxalic acid, and the colony was fed a home-made sucrose solution (3:2 sucrose/water) in fall; no other supplements were added to the hive.

The queen, acquired from a commercial queen breeder and introduced to the colony in 2018, remained during the whole duration of the study. The brood rearing pattern of the colony was consistent with that of Italian bees in a southern temperate climate with only a short cessation (approximately 2 weeks) in mid-winter. No foraging stop was observed. The colony was housed in a Dadant hive with commercial brood frames with a cell size of 5.4 mm.

Ten forager bees were collected on a monthly basis (on the last week of each month; e.g. Sauthier et al., 2017), for a total of 70 individuals. Bees returning to the hive were captured from the flight deck using a horsehair brush in order to ensure the exclusion of young bees. Captured bees were stored in sterile glass containers (250 cl) with breathable fabric lid and transported to the laboratory of animal production and nutrition of the university of Sassari (UNISS). Individual bees were weighed using a digital scale having a precision of 0.01 g (OHAUS® PA512C) before being subjected to freezing at -18°C for 48 h, and stored until further analysis. Any pollen or visible attachments were removed manually prior to weighing.

All operations carried out on live animals were performed by trained veterinarians and the study was carried out following the recommendations of the European Council Directive (86/609/EEC) on the protection of animals.

2.2. Morphological analysis

Sampled bees were analysed in their entirety and within a frozen state insuring correct proportional morphological retention. Using a digital caliper (precision 0.01 mm) under a stereomicroscope (Leica® EZ4 HD), the total body length, the width and length of the abdomen and thorax, and the width of the head were measured for each individual bee. Width measurements of each respective body part were taken at the widest point. Length measurement of the thorax and abdomen were taken from the anterior end of the protergum to the caudal end of the first abdominal tergum (T_1-IT; T_1 includes the scutum and scutellum) and the anterior end of the second abdominal tergum to the caudal end of seventh abdominal tergum (IIT-VIIT) not including the stinger, respectively.

2.3. Fat body quantification

An estimation of the weight and relative size of the fat body was performed for each individual bee applying the ether extraction method according to Wilson-Rich et al. (2008). Briefly, abdomens of bees were severed using surgical scissors and placed into individual holding cups to dry at room temperature (25°C; using a Binder ED 53 drying oven) for 3 days. Next, abdomens were submerged in diethyl ethyl ether for 24 h and then dried again for 3 days (same conditions). Severed, dried abdomens were weighed (using a ORMA BCA200 electric laboratory balance, precision 0.0001 g) before and after ether extraction. The fat body weight was calculated as the difference in abdomen weight before and after washing with diethyl ether and the relative size of the fat body as the proportional weight of the fat body relative to the weight of the dried abdomens prior to ethyl ether extraction (Strachecka et al., 2021; Wilson-Rich et al., 2008).

2.4. Data analysis

Pearson's test for the assessment of potential correlation between individual bee bodyweight, respective body, and fat body measures ($\rho < 0.300$ = weak correlation, $0.300 < \rho < 0.600$ = mild correlation, $0.600 < \rho < 1.000$ = strong correlation, $+\rho$ or $-\rho$: positively or negatively correlated, respectively; significance for p-value < 0.05) was used.

Analysis of variance (ANOVA) was performed to detect any changes in individual weight, selected morphological metrics, and fat body parameters of sampled bees according to sampling date. All procedures were carried out using a software package (Minitab statistical software package, Minitab©, New York, NY, USA). Effects were evaluated by pairwise comparison by using the Tukey multiple comparison test. Statistical significance was set at p-value <0.05.

3. Results

Overall, sampled forager bees had a mean body weight (BW) of 0.07 g (SD: 0.01), mean abdominal length (AL) of 5.23 mm (SD: 0.60), mean abdominal width (AW) of 4.14 mm (SD: 0.17), mean thoracal length (TL) of 3.75 mm (SD: 0.05), mean thoracal width (TW) of 3.75 mm (SD: 0.04), mean head width (HW) of 3.68 mm (SD: 0.07), and a mean total body length (T) of 11.29 mm (SD: 0.49). Sampled bees had a mean fat body weight (FBW) of 0.77 mg (SD: 0.41) and a mean proportional fat body size (FB%) of 10.98% (SD: 4.03%). Summarizing weight, morphometrical, and fat body quantification data of sampled bees per month are reported in Table 1.

Table 1. Summarising weight, morphometrical, and fat body quantification data of forager honey bees from Sassari (Italy) according to sampling month.

Month/Variable	*November*	*SD*[a]	*December*	*SD*[a]	*January*	*SD*[a]	*February*	*SD*[a]	*March*	*SD*[a]	*April*	*SD*[a]	*May*	*SD*[a]
Body weight	0.07	0.01	0.09	0.02	0.07	0.01	0.07	0.01	0.07	0.01	0.07	0.01	0.07	0.01
(range, g)	(0.06-0.10)		(0.07-0.13)		(0.06-0.09)		(0.05-0.07)		(0.06-0.08)		(0.06-0.08)		(0.06-0.09)	
Abdominal length	5.18	0.35	5.95	0.92	5.10	0.19	5.05	0.41	5.08	0.66	5.13	0.28	5.13	0.58
(range, mm)	(4.75-5.75)		(4.84-7.98)		(4.86-5.44)		(4.23-5.71)		(3.87-5.98)		(4.62-5.48)		(4.28-6.31)	
Abdominal width	4.17	0.12	4.30	0.23	4.09	0.15	4.09	0.14	4.11	0.15	4.10	0.19	4.13	0.09
(range, mm)	(3.97-4.42)		(4.01-4.69)		(3.81-4.31)		(3.81-4.31)		(3.87-4.38)		(3.82-4.44)		(4.00-4.27)	
Thoracal length	3.76	0.04	3.77	0.05	3.72	0.05	3.72	0.06	3.74	0.05	3.76	0.05	3.76	0.05
(range, mm)	(3.70-3.82)		(3.66-4.83)		(3.64-3.80)		(3.64-3.80)		(3.65-3.80)		(3.68-3.82)		(3.70-3.84)	
Thoracal width	3.75	0.06	3.76	0.04	3.73	0.04	3.73	0.05	3.76	0.05	3.75	0.03	3.76	0.04
(range, mm)	(3.66-3.84)		(3.70-3.80)		(3.65-3.79)		(3.64-3.80)		(3.66-3.82)		(3.70-3.79)		(3.67-3.81)	
Head width	3.67	0.07	3.77	0.10	3.65	0.04	3.65	0.06	3.65	0.06	3.67	0.05	3.68	0.04
(range, mm)	(3.59-3.75)		(3.60-3.90)		(3.59-3.72)		(3.55-3.74)		(3.51-3.75)		(3.59-3.75)		(3.62-3.72)	
Total body length	11.21	0.28	11.82	0.66	11.14	0.27	11.13	0.24	11.26	0.47	11.27	0.53	11.19	0.55
(range, mm)	(10.63-11.56)		(11.08-13.29)		(10.88-11.61)		(10.65-11.41)		(10.30-11.81)		(10.45-11.87)		(10.48-12.03)	
Fat body weight	0.71	0.47	1.18	0.70	0.71	0.28	0.63	0.24	0.65	0.16	0.71	0.27	0.78	0.35
(range, mg)	(0.30-1.70)		(0.60-2.90)		(0.10-1.10)		(0.40-1.20)		(0.50-0.90)		(0.30-1.30)		(0.10-1.20)	
Fat body size	10.52	5.14	15.76	4.30	10.93	3.31	8.46	1.95	10.36	2.28	10.49	3.10	10.37	4.01
(range, %)	(5.26-18.84)		(9.68-22.73)		(2.17-13.33)		(5.26-11.77)		(8.07-15.25)		(5.06-15.48)		(1.89-15.71)	

[a] Standard Deviation.

Results of the Pearson's test for the assessment of potential correlation between individual bee body weight, respective body, and fat body measures are reported in Table 2.

Table 2. Results of Pearson correlation analysis between individual bee body weight, respective body, and fat body measures of forager bees from Sassari (Italy).

	Weight	***Head width***	***Thoracal width***	***Thoracal length***	***Abdominal width***	***Abdominal length***	***Total body length***	***Fat body weight***
Head width	0.432^{**}	/	/	/	/	/	/	/
P-value	<0.001	/	/	/	/	/	/	/
Thoracal width	0.110	0.528^{**}	/	/	/	/	/	/
P-value	0.366	<0.001	/	/	/	/	/	/
Thoracal length	0.191	0.668^{***}	0.797^{***}	/	/	/	/	/
P-value	0.113	<0.001	<0.001	/	/	/	/	/
Abdominal width	0.599^{**}	0.411^{**}	0.341^{**}	0.292^{*}	/	/	/	/
P-value	<0.001	<0.001	<0.001	0.014	/	/	/	/
Abdominal length	0.858^{***}	0.416^{**}	0.010	0.080	0.470^{**}	/	/	/
P-value	<0.001	<0.001	0.935	0.513	<0.001	/	/	/
Total body length	0.799^{***}	0.366^{**}	0.109	0.090	0.598^{**}	0.811^{***}	/	/
P-value	<0.001	<0.001	0.370	0.459	<0.001	<0.001	/	/
Fat body weight	0.699^{***}	0.349^{**}	-0.059	0.112	0.418^{**}	0.643^{***}	0.537^{**}	/
P-value	<0.001	0.003	0.627	0.357	<0.001	<0.001	<0.001	/
Fat body size	0.600^{***}	0.313^{**}	-0.082	0.061	0.299	0.498^{**}	0.422^{**}	0.848^{***}
P-value	<0.001	0.004	0.502	0.614	0.012	<0.001	<0.001	<0.001

*indicates a weak correlation.

**indicates a mild correlation.

***indicates a strong correlation.

Analysis of variance showed a significant difference in BW, AL and HW throughout the sampling months ($F_{(6, 63)} = 5.36$, $p < 0.001$; $F_{(6, 63)} = 3.47$, $p = 0.005$; $F_{(6, 63)} = 4.67$, $p = 0.001$; Figure 1) and *post hoc* analysis revealed bees present in December to have greater values for all three mentioned parameters as compared to the rest of the months. Abdominal width and T of bees were found to differ based on sampling time as well ($F_{(6, 63)} = 2.31$, $p = 0.044$; $F_{(6, 63)} = 2.83$, $p = 0.017$), although Tukey test revealed no decisive patterns. Sampling time was not found to have any effect on TL and TW. ANOVA revealed a significant difference in FBW and FB% size over the sampling months as well ($F_{(6, 63)} = 2.34$, $p = 0.042$; $F_{(6, 63)} = 3.93$, $p = 0.002$; Figure 1.). *Post hoc* analysis showed bees sampled in the month of December to have significantly larger FB% compared to the rest of the months although no clear difference in FBW between months was pointed out.

Figure 1 shows the interval plots for BW, AL, HW, and FB% according to sampling month.

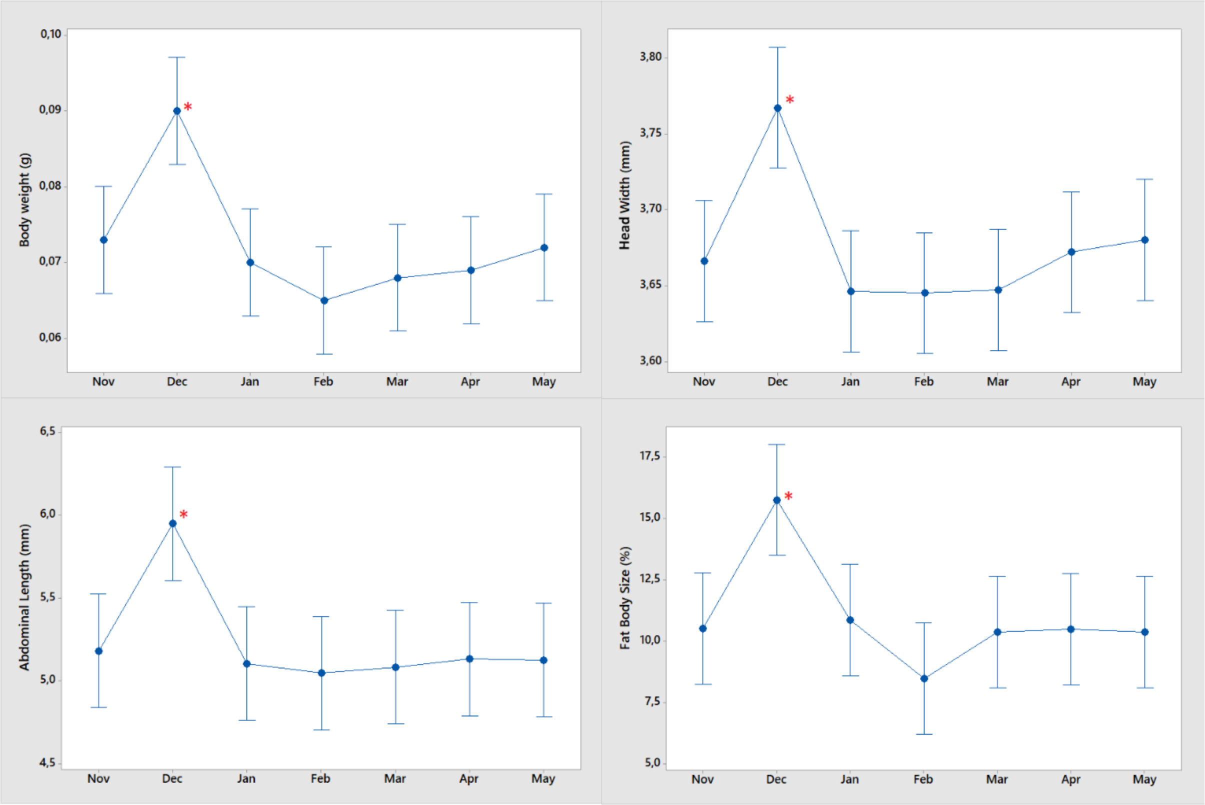

Figure 1. Interval plots of body weight, abdominal length, head width, and proportional fat body size according to sampling month. Red* indicates months significantly different for unmarked months. Nov= November, Dec= December, Jan= January, Feb= February, Mar= March, Apr= April.

4. Discussion

In this research, a preliminary evaluation of novel, easy-to-use morphological metrics of worker honey bees; Abdominal width (AW), Abdominal length (AL), Thoracal width (TW), Thoracal length (TL), Head width (HW), and Total body length (T) was carried out through correlation analysis with known markers of honey bee health and nutrition; Body weight (BW), Fat body weight (FBW), and Proportional fat body size (FB%). Additionally, as a proof of concept, selected metrics were monitored through monthly sampling of a single colony in order to detect possible size variations corresponding to temporal changes in the nutritional status of honey bees. This approach was chosen as honey bee workers from temperate climates of the northern hemisphere exhibit significant fluctuations in their nutritional status with a specific seasonal pattern (Knoll et al., 2020). Authors elected to sample forager (out-hive) bees rather than nurse (in-hive) bees for two main reasons; 1) less individual variation in nutrient storage can be expected between forager bees, and 2) seasonal variation of body traits of forager bees is likely to be low (Sauthier et al., 2017), providing higher sensitivity to the selected markers. These assumptions are based on the fact that the age division of labour among worker honey bees is closely related to nutrient storage (fat and protein): high nutrient stores of young bees (who are confined to in-hive tasks) generally decrease with age and depletion of these stores marks the onset of foraging (Amdam and Omholt, 2002; Toth et al., 2005; Toth and Robinson, 2005; Ament et al., 2010). The recommended minimum sample size of 10 uniform bees per hive for morphometrical analysis was followed (Ruttner, 1988; Meixner et al., 2013).

Records of robust morphological measurements of honey bees are generally lacking in the scientific literature. This is likely due to the high size variability between honey bee subspecies, populations, and even colonies, and thus publication of such measurements to serve little function (Ruttner, 1988; Sauthier et al., 2017). Furthermore, size variation between worker honey bees of a single colony is considered to be negligible (Kerr and Hebling, 1964; Roulston and Cane, 2000; Chole et al., 2019). However, it is not clear if temporal (e.g. seasonal) differences were taken into account when formulating this statement. For example, Sauthier et al. (2017) showed a significant size difference between forager bees over the course of the foraging season in two out of three sampled colonies. For as far as comparison is possible, mean measurements of size obtained in this research correspond to previously published data (Kerr and Hebling, 1964; Ruttner, 1988; Sauthier et al., 2017).

Body weight is a known marker for the nutritional status of honey bees and is closely related to the size of the fat body, the main nutrient storage tissue in bees (Amdam and Omholt, 2002; Toth and Robinson, 2005; Brodschneider and Crailsheim, 2010; Ament et al., 2011; Smart et al., 2016; Kunc et al., 2019; Ricigliano et al., 2019; Koubová et al., 2021). This is supported by our findings showing a strong positive correlation between BW and FBW and a mild positive correlation between BW and FB%. Given the strong correlation between FBW and FB%, the mild correlation between BW and FB% is somewhat counterintuitive. Nevertheless, this can be explained by the fact that fat body cells are not limited to the abdomen but are also found (to a lesser extent) in the thorax and head of bees and that FB% is calculated as the proportional weight of the abdominal fat body compared to the overall weight of the abdomen specifically (Corona et al., 2007; Seehuus et al., 2007). Furthermore, FB% is strongly affected by the foraging activity of a particular bee as the FB% of foragers with a full honey stomach will be less proportional to their BW (Toth and Robinson, 2005). For this reason, FBW can be considered a better reflection of the nutritional status of foragers than FB%.

The results of the present research show a strong correlation between AL and T *Vs.* BW and FBW as well. This is logical as fat body tissue is primarily located in the abdominal cavity of bees (Corona et al., 2007; Seehuus et al., 2007). Once again, one could argue that the positive correlation between AL (as well as T) and BW to be the result of foraging activities and thus reflect honey bees returning to the hive with a full nectar load. While this could be the case for some individual samples (as is shown by the mild correlation between AL and FB%), this would not explain the strong significant correlation between AL and FBW seen here. In fact, AL and BW showed the highest correlation of all metrics in this research. Hence, a well-developed fat body could result in measurable morphological variations in worker honey bees, possibly serving as a marker for their nutritional status. Correspondingly, the strong correlation seen between AL and T is self-explanatory.

The TW and TL of forager bees were shown to be strongly positively correlated to each other, which again is rather intuitive. These metrics are probably relatively constant regardless of nutritional status. This is evidenced by the overall low monthly variation (SD: Table 1) in the present data and the lack of/or generally weak correlation of these metrics with measurements found to be more representative of honey bee nutrition (W, T, AL, FBW, FB%).

Surprisingly, BW, FBW, AL, and T were only mildly positively correlated to the AW of sampled bees. A weak positive correlation was also shown for AW *Vs.* FB%. It is probable that the anatomy of the honey bee abdomen, with its consecutive partially overlapping tegra, allows for longitudinal rather than horizontal expansion and accounts for these findings. Authors argue a higher correlation between AW and FBW and/or FB% could be seen in in-hive bees where a substantially larger fat body (Amdam et al., 2003; Toth and Robinson, 2005) may allow for a width expansion of the abdomen. Alternatively, the use of more precise measuring methods could reveal more decisive patterns. Regardless, it seems the nutritional status of bees is better reflected by the overall length of the abdomen rather than by its width, at least in foragers.

Finally, even though HW is reported to be well correlated to BW (Sauthier et al., 2017), HW was consistently mildly correlated to metrics related to honey bee nutrition discussed above. From a nutritional point of view, HW of worker bees could be affected by two factors; 1) fat body tissue in the head (Corona et al., 2007; Seehuus et al., 2007), and 2) HPG size [a known marker of honey bee nutrition due to its assumed nutrient storage function (Fluri et al., 1982; Crailsheim, 1990; Amdam and Omholt, 2002; Mattila and Otis, 2007; Degrandi-hoffman et al., 2010)]. Given that forager bees have hypotrophied HPGs (Amdam and Omholt, 2002; Döke et al., 2015; Ali et al., 2019) and depleted nutrient reserves, it is not surprising HW to be a mediocre reflection of the nutritional status of forager bees. Furthermore, the strong correlation of this metric with TL and TW, as well as its low monthly variability (except for December, see Table 1), indicates this metric to be rather stable in forager bees.

The monitoring of selected metrics over the transition period of autumn-winter-spring (2019-2020) revealed significant size variations of forager bees across sampling months. Abdominal length and HW were shown to be the most promising temporal markers for the nutritional status of foragers, exhibiting corresponding fluctuations with BW and FB%. Specifically, a significant increase in AL, HW, BW, and FB% was shown for the month of December (Figure 1). The size difference between bees sampled in December compared to other months is illustrated in Figure 2.

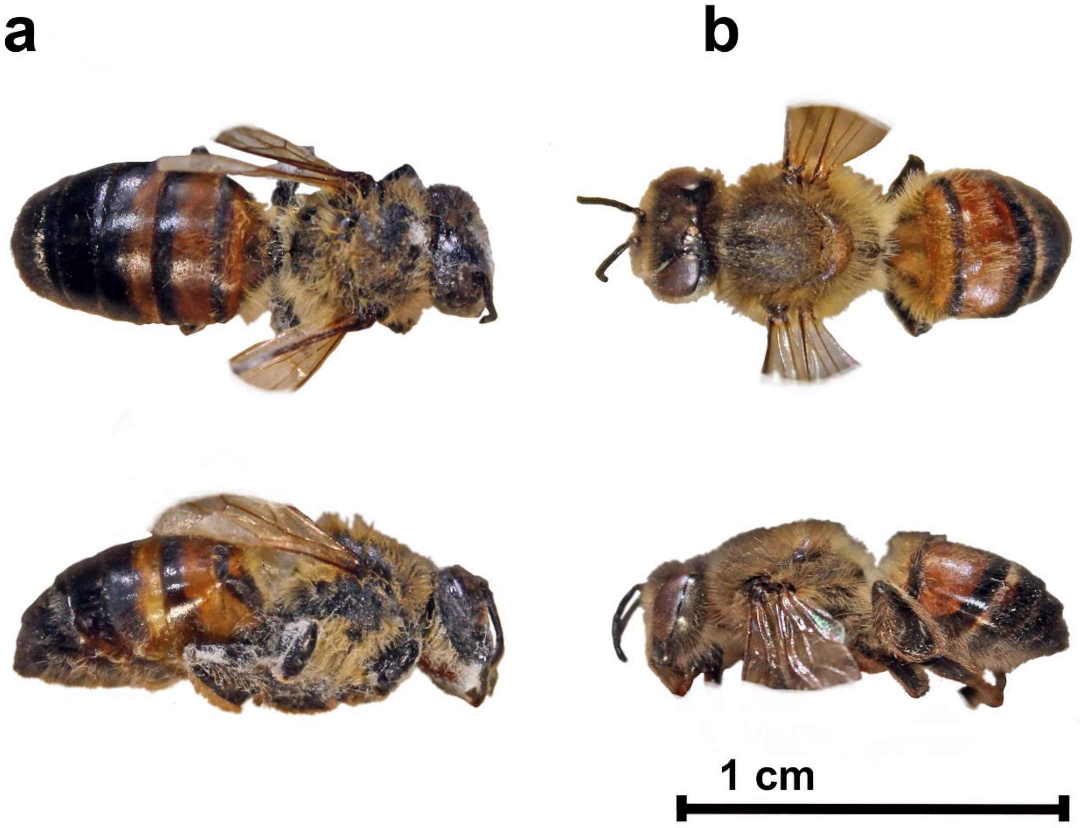

Figure 2. Illustrative image of the notable morphological difference in abdomen size of forager honey bees according to sampling month. (a) December and (b) rest of the sampling months.

Forager bees with increased size in December were identified to be winter bees exiting their overwintering state (Alaux et al., 2018). Indeed, in a Mediterranean climate, relatively high temperatures in winter result in an early start of the brood rearing season. Furthermore, in such a climate *Apis m. ligustica* is known to exhibit only a short cessation of activities (Ruttner, 1988; Gupta et al., 2014). This is in accordance with the findings of Sauthier et al. (2017) who recorded forager bees with larger morphological dimensions in early spring likely to be winter bees hatched the year before. The differences in AL, HW, BW, and FB% observed in forager bees in this research would thus be remnants of the nutrient accumulation that occurred during in-hive activities.

Significant differences in T, AW, and FBW between months were found as well, although *post hoc* analysis revealed no discriminating pattern. Nevertheless, interval plots of AL, HW, T, AW, FBW, and FB% showed a corresponding pattern with a main peak in December. It is suspected the decrease in nutrient storage preceding the foraging stage resulted in worker bees with uniform FBW leading to the absence of a decisive temporal pattern in our samples.

Interestingly, HW was found to be a suitable candidate indicator for the presence of winter bees while this metric was shown to be only mildly correlated to nutrition in foragers. This probably results from the fact that, contrary to in-hive bees during the rest of the year, winter bees have hypertrophied HPGs (Amdam and Omholt, 2002; Amdam et al., 2003; Mattila and Otis, 2007; Seehuus et al., 2007). Authors hypothesize a significant increase in HPG size in winter bees might cause enlargement of their head capsule which is still notable in spring.

Lastly, it is worth pointing out that monthly SDs of selected metrics (except of TL and TW) revealed a pattern in line with current knowledge of the honey bee colony cycle in temperate climates of the northern hemisphere. Specifically, the transition of a colony from its summer to its winter state and back is gradual. Therefore, during transition months, a mixed population of summer and winter bees is present within a hive (Knoll et al., 2020). This is reflected by the roughly doubled SD seen for most selected metrics in December (Table 1).

Findings of this research illustrate the potential of robust measurements of individual size as markers for the nutritional status of honey bees across temporal patterns, with several metrics showing more promise than others. Candidate markers identified in this preliminary assessment could offer considerable advantages in the context of research efforts related to global colony losses and the health monitoring of honey bees in general. Firstly, performing size measurements is technically easy, requires no specialized equipment nor training, and is cost-effective. This is in contrast with the analysis of many commonly employed (molecular and biochemical) markers for individual honey bee health which entail extensive laboratory analysis (Smart et al., 2016; Kunc et al., 2019; López-Uribe et al., 2020). A second potential advantage is the application of size measurements as non-invasive metrics in the real-time monitoring of honey bee hives. For example, a smart system of in-hive monitoring of individual honey bee size through automated sensors [e.g. camera system placed at the entrance of a hive (Ammar et al., 2019; Sevin et al., 2021)] and machine learning could be envisioned (Lofaro, 2017; Hong et al., 2020). Furthermore, individual worker size variations between honey bee hives would not pose an issue in such a scenario as machine learning could distil dynamic size patterns regardless of the baseline size of bees from a specific colony. Additional input (e.g.

queen replacement, queen age, subspecies, genetic background, drone recognition, …) and the enrichment of such a system with added sensors [e.g. overall weight of the hive, temperature, humidity, … (Cecchi et al., 2020)] can provide added sensitivity to potential markers.

Regardless, further research efforts are required to validate candidate markers as representative of the health and nutritional status of honey bees. To this end, various steps are recommended; 1) Selected metrics should be evaluated in in-hive bees as well in order to assess temporal size variations in this nutritionally distinct type of worker bee; 2) Given genetic variability between honey bee populations, evaluation of selected metrics should be performed on various honey bee subspecies; 3) Since this research was performed on a single hive and over a restricted period, long-term evaluation with a larger sample size and over various spatial and geographical variations is advisable; 4) Experimental research investigating the sensitivity of potential markers for the detection of health issues rather than physiological variations should be conducted.

This research provides basic knowledge on size variations of honey bee foragers over temporal patterns. Our findings can be used as a cornerstone for future research evaluating size metrics as possible markers for honey bee health and nutrition.

5. Conclusion

Significant temporal differences in worker honey bee size correlated to nutrition were highlighted in this research. Results revealed abdominal length in particular to be a suitable candidate marker for the assessment of the health and nutritional status of forager bees. While total body length performed less well in the “proof of concept”, this metric had a strong positive correlation with abdominal length, body weight, and fat body size. Contrary, head width was not shown to be strongly correlated to nutrition in foragers, but remnants of in-hive physiology could make this a useful marker over temporal patterns. Other selected morphological metrics showed less promise, although require further investigation. Overall, further research efforts are necessary to explore the full potential of candidate markers.

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DECLARATION OF COMPETING INTEREST

Authors declare that no conflict of interest exists.

AUTHOR CONTRIBUTION

Stephane Knoll: data collection and analysis, and paper writing; Valeria Fadda: data collection; Fahad Ahmed: manuscript review; Maria Grazia Cappai: project design, data analysis and manuscript review.

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CHAPTER 4

The nutritional year-cycle of Italian honey bees (*Apis mellifera ligustica*) in a southern temperate climate

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Highlights

- Long-term nutritional monitoring of Italian honey bees in a southern temperate climate (Sassari, Italy) was conducted and data analysed in function of sampling date, climatological factors (temperature, precipitation, and daylength), and flower diversity.
- The short presence of winter bees, characterised by increased nutrient storage and reduced brood rearing, was shown in late fall/early winter.
- Honey bee nutrition generally followed the nectar flow and showed two critical timepoints: summer and winter dearth.
- Precipitation was found to play an important role in honey bee nutrition through its impacts on plants in particular illustrating how climate change could pose a threat to European honey bee populations in the future.

Abstract

Nutrition is a key aspect influencing honey bee health and overwintering. Since seasonal activity of honey bees in southern temperate climates represents a significant research gap, this study conducted long-term monitoring of honey bees in the Mediterranean (Sassari, Italy). Specifically, individual weight, fat body, and size measurements (head, thorax, abdomen, and total body) were recorded monthly (100 bees; 5 hives) in order to detect changes in the nutrient storage of worker bees during an annual cycle (2022-2023). Recorded parameters were analysed according to sampling date, climate (temperature, precipitation, and daylength), and flower diversity and was conducted for nurse and forager bees separately. Additionally, size metrics were compared between cohorts, showing significant differences for all measurements except for the thorax. The nutritional honey bee year-cycle generally followed the nectar flow and showed two critical timepoints: summer and winter dearth. A short cessation of activities in late fall/early winter coupled with an increase in nutrient storage and in opposition to flower diversity indicated the presence of winter bees. Precipitation was found to play an important role in honey bee nutrition through its impacts on colony demography and plants in particular illustrating how climate change could pose a threat to European honey bee populations in the future. All in all, our results provide valuable insights into the nutritional dynamics of *Apis mellifera ligustica* in the Mediterranean that could support more effective management decisions in relation to environmental factors and availability of flowering plants with the goal of improving overwintering success and preventing unnecessary colony losses.

KEYWORDS

Winter bees, Nutrition, Morphometry, Southern temperate climate, Climate change

1. Introduction

Honey bee colony losses represent a grave and yet relatively poorly understood issue in modern apiculture (Jacques et al., 2017; Beyer et al., 2018; López-Uribe et al., 2020; Bruckner et al., 2022; Insolia et al., 2022; Mutinelli et al., 2022). Regardless of climate, most losses occur in winter, which is a particularly challenging period for these social insects as there is little to no natural forage available (Döke et al., 2015; Kunc et al., 2019; Gray et al., 2022).

Historically, as honey bees (*Apis mellifera* Linnaeus, 1758) spread from tropical/subtropical regions to temperate climates of the northern hemisphere they evolved unique adaptations allowing colonies to bridge harsh winter conditions without entering a dormant state (Han et al., 2012; Wallberg et al., 2014; Amdam et al., 2005; Chen et al., 2016). Specifically, honey bees synchronized their activities with plant phenology, greatly reduced brood rearing in winter, and assumed the formation of a thermoregulating cluster during the coldest months (Mattila et al., 2001; Döke et al., 2015; Kunc et al., 2019). Furthermore, *A. mellifera* adopted significant seasonal changes in individual lifespan within its yearly cycle. This has led to the description of two temporally distinct worker bee types; while the honey bee workforce is made up of classical short-lived “summer bees” during most of the year, in winter, these bees are replaced by long-lived winter or *diutinus* bees (Knoll et al., 2020).

Besides assuring colony survival through thermoregulation (Döke et al., 2015; Nürnberger et al., 2018), winter bees effectively function as a “nutrient storage caste” (Amdam and Omholt, 2002; Amdam et al., 2003; 2005). These bees store large amounts of fat and protein within their bodies (through the accumulation of vitellogenin: Vg) which are conserved throughout winter and subsequently utilized to reinitiate brood rearing when the return of favourable environmental conditions is anticipated (Münch et al., 2013; Kunc et al., 2019). Moreover, it is this same Vg that grants winter bees their longevity (Amdam and Omholt, 2002; Amdam et al., 2004; Seehuus et al., 2006; Corona et al., 2007), illustrating the fundamental role of nutrition for the survival of cold-adapted honey bees. Other typical features of *diutinus* bees (hypertrophied hypopharyngeal glands, enlarged fat bodies, and elevated hemolymph protein contests) are also related to nutrition (Mattila et al., 2001; Amdam and Omholt, 2002; Mattila and Otis, 2007; Döke et al., 2015; Van der Steen et al., 2015; Kunc et al., 2019; Koubová et al., 2021).

Extensive research has allowed for the description of an elegant system showing how honey bees in temperate zones have adapted mechanisms of age division of labour into a bimodal, biannual worker caste system governed by a multitude of internal and external factors with varying sensitivity (*reviewed in* Knoll et al., 2020). In brief, deteriorating environmental conditions and the disappearance of nutrient resources (nectar and pollen) likely cause a drastic reduction in brood rearing, triggering the transition of newly emerging bees into *diutinus* bees. It is noteworthy that this seasonal shift is mainly linked to the dwindling pollen availability in fall rather than to fluctuations in meteorological factors offering temporal plasticity and adaptability in a changing climate (Mattila and Otis, 2007).

Whereas overwintering of honey bees in northern regions has been well studied (Döke et al., 2015; Kunc et al., 2019; Knoll et al., 2020), much less is known regarding the seasonal dynamics of these insects in southern temperate climates. At these latitudes, warm summers and soft winters generally allow for a long foraging season and only a short cessation of activities in winter (Ruttner, 1988; Keller et al., 2005b; Ricigliano et al., 2018; Maes et al., 2021). While this seems advantageous, relatively high winter temperatures can lead to unsustainable brood rearing causing exhaustion of worker bees towards spring (Ruttner, 1988; Nürnberger et al., 2018). Moreover, extended periods of foraging resource dearth (e.g. during summer droughts) can put nutritional stress on a colony, hampering preparations for winter (Le Conte and Navajas, 2008; Russell et al., 2013; Ricigliano et al., 2018; Hristov et al., 2021).

Inadequate nutrition has been identified as a dominant factor in honey bee colony losses (Goulson et al., 2015; Kim et al., 2022) and has been shown to have significant effects on individual and colony health and development, including colony size, lifespan, immunity, and overwintering success (Keller et al., 2005a; 2005b; Naug, 2009; Alaux et al., 2010; Brodschneider and Crailsheim, 2010; Huang, 2012; Goulson et al., 2015; Di Pasquale et al., 2016; Filipiak et al., 2017; Dolezal et al., 2018; Ptaszyńska et al., 2021; Retschnig et al., 2021). Moreover, poor nutrition increases the sensitivity of honey bees to other stressors (e.g. pests and diseases) and other stressors (e.g. agricultural intensification and climate change) contribute to malnutrition of honey bee colonies (De La Rúa et al., 2009; Neumann and Carreck, 2010; Goulson et al., 2015; Scofield et al., 2015; Potts et al., 2016; Steinhauer et al., 2018; Watkins de Jong et al., 2019; Kim et al., 2022; Castle et al., 2023).

Lastly, research efforts in relation to seasonal adaptations of honey bees have mainly focussed on northern subspecies (e.g. *Apis mellifera mellifera*) (Chen et al., 2016). Since a higher survival rate of locally adapted subspecies (Meixner et al., 2015) as well as adaptation to specific climatic conditions (Büchler et al., 2014; Dražić et al., 2014; Kovac et al., 2014) has been shown, knowledge of southern honey bee populations is of increasing interest, especially in the face of accelerated climate change the world is facing in present days (Le Conte and Navajas, 2008; Espregueira et al., 2020).

Against this background, this study aimed to conduct long-term monitoring of the nutritional status of locally adapted Italian honey bees (*Apis mellifera ligustica*, Spinola 1806) in a Mediterranean climate (Sassari, Italy). The goal of this research was to provide a better understanding of the activity and nutritional status of worker (both nurse and forager) bees in southern temperate climates and to generate new insights on the dynamics of the summer and winter bee transition in correlation with seasonal changes in environmental factors and feed resource availability. In addition, authors aimed to provide novel knowledge regarding the seasonal dynamics of Italian honey bees specifically, and the possible challenges these bees face in a changing climate.

2. Materials and methods

2.1. Study site and Apiary

Monitoring was conducted between February 2022 and January 2023 (12 months). Colonies of Italian honey bees (*Apis mellifera ligustica* Spinola, 1806), located in a private apiary in the province of Sassari (Sardinia, Italy; 40°37'14.5"N 8°20'43.1"E), were studied. The southern temperate Mediterranean climate of the study area, with hot dry summers and mild wet winters, typically allows for a long foraging season and only a short cessation of activities in winter. The initiation of the study was planned according to the seasonal pattern of Italian honey bees in the region, coinciding with the start of the foraging season.

Meteorological data over the course of the study, including mean monthly temperature, precipitation, days-count with precipitation, relative humidity, windspeed, and daylength (hours of daylight), as retrieved from the weather station of the meteorological services of the Military Airforce of ENAV (Ente Nazionale Assistenza al Volo) located approximately 15 km from the study area are summarized in Table 1.

Table 1. Summarizing meteorological data for the province of Sassari (Italy), February 2022 - January 2023.

	Temperature (°C)		*Precipitation (mm)*		*Precipitation (Days)*	*Wind (Km/h)*	*Humidity (%)*	*Daylength (min)*	
Month*		***Category*****		***Category*****					***Category*****
Jan**	10.4	***Ta***	83.4	***Pb***	17	11.0	82	575	***Oa***
range	-1.0-21.0		/		/	0-45.0	60-98	/	
Feb	10.3	***Ta***	19.3	***Pa***	4	11	77	641	***Oa***
range	-2.0-18.0		/		/	0-40.0	53-96	/	
Mar	10.8	***Ta***	33.7	***Pa***	12	11.2	71	728	***Ob***
range	-1.0-22.0		/		/	0-33.8	46-93	/	
Apr	14.2	***Ta***	61.5	***Pb***	8	14.0	70	813	***Oc***
range	3.0-25.0		/		/	0-45.0	44-95	/	
May	19.6	***Tb***	89.7	***Pb***	6	9.0	74	877	***Od***
range	7.0-34.0		/		/	0-35.0	45-99	/	
Jun	25.0	***Tc***	3.8	***Pa***	4	9.9	66	903	***Od***
range	13.0-39.0		/		/	0-34.0	35-95	/	
Jul	26.7	***Td***	0.3	***Pa***	0	8.9	66	893	***Od***
range	12.0-39.0		/		/	0-32.0	33-97	/	
Aug	26.8	***Td***	12.9	***Pa***	3	10.0	72	822	***Oc***
range	17.0-37.0		/		/	0-37.0	42-97	/	
Sep	23.3	***Tc***	108.6	***Pc***	9	7.0	75	730	***Ob***
range	12.0-35.0		/		/	0-48.0	48-98	/	
Oct	19.5	***Tb***	46.6	***Pa***	6	7.5	81	654	***Ob***
range	9.0-29.0		/		/	0-29.0	50-100	/	
Nov	15.5	***Tb***	148.4	***Pc***	16	9.9	82	597	***Oa***
range	5.0-24.0		/		/	0-45.0	60-99	/	
Dec	13.2	***Ta***	144.5	***Pc***	11	10.7	88	559	***Oa***
range	4.0-20.0		/		/	0-37.0	67-100	/	

*Jan = January, Feb = February, Mar = March, Apr = April, Jun = June, Jul = July, Aug = August, Sep = September, Oct = October, Nov = November, and Dec = December.

**Data for the month of January were collected in 2023 while the rest of the months regard the year 2022.

***Categories of environmental factors for statistical analysis: 1) monthly average ambient temperature is divided into 4 levels; *Ta*, *Tb*, *Tc*, *Td*; range: 10-15, >15-20, >20-25, >25 °C. 2) mean monthly precipitation is divided into 3 levels; *Pa*, *Pb*, *Pc*; range: 0-50, >50-100, >100 mm. 3) monthly average daylength was divided into 4 levels; *Oa*, *Ob*, *Oc*, *Od*; range: 550-650, >650-750, >750-850, >950 h of daylight.

Hives were located in a semi-natural agricultural area, surrounded by managed and unmanaged fields, vineyards, olive groves, and small-scale mixed agriculture (vegetable gardens). The botanical composition of spontaneous flora and the phenological state of plants, with particular regard to pollen availability, in the direct vicinity of the apiary, was monitored throughout the sampling period. Specifically, in order to assess the diversity of flowering plants, 3 100x2 m transects were defined prior to the initiation of the study. At each sampling date, the 3 transects were walked by a single observer and the various species of flowering plants known to be visited by honey bees recorded. Honey bee samples were collected from 5 individual hives selected by the responsible apiarist based on overall health and uniformity. Selected colonies were separated from the rest of the apiary by a distance of 25 m before the initiation of the study. Colonies received standardized care during the study period and were inspected weekly insuring good health. No clinical signs of disease were noted during the course of this study. Treatment against the ectoparasitic mite *Varroa destructor* was applied in March, August, and December using Amitraz and oxalic acid. Colonies were fed a homemade sucrose solution (3:2 sucrose/water) in spring and fall. No other nutritional supplements were provided.

Apis m. ligustica queens were acquired from a commercial queen breeder and introduced to each respective colony the year before. Queens remained during the whole duration of the study. Colonies were housed in wooden Dadant-type hives with 10 commercial brood frames with a cell size of 5.4 mm.

Brood rearing patterns were consistent with that of Italian bees in a southern temperate climate [exhibiting a “Mediterranean pattern” as has been described for bees on the neighbouring island of Corsica (Gupta, 2014)]; showing a steady increase from spring until peaking in June and subsequently decreasing during hot summer months. A second minor peak was seen in early fall. Three out of the five hives showed a cessation of brood rearing (for approximately 2 weeks) in early December and brood rearing remained relatively low until spring. No foraging stop was observed for any of the hives.

2.2. Sample collection

Ten forager and ten nurse bees from each hive were collected separately on the last week of each month (100 individuals; 50 foragers/50 nurse bees per month). 1) Foragers: bees returning to the hive were captured from the flight deck using a horsehair brush. 2) Nurse bees: young bees from the centre of the brood nest were collected. Captured bees were stored in 250 cl glass containers with breathable fabric lids and transported to the laboratory of animal production and nutrition of the university of Sassari (UNISS) in a cooler box with icepacks. Individual bees were weighed using a digital scale (OHAUS® PA512C; precision of 0.01 g) before being frozen (-18 °C) and stored in 1.5 ml microcentrifuge tubes until further analysis. Any pollen or visible attachments were removed manually prior to weighing.

2.3. Morphological analysis

Sampled bees were analysed in their entirety and within a frozen state insuring correct proportional morphological retention. Using a digital calliper (precision 0.01 mm) under a stereomicroscope (Leica® EZ4 HD), six size measurements were taken for each individual bee; 1) Head width (HW), 2) thoracal width (TW), 3) thoracal length (TL), 4) abdominal width (AW), 5) abdominal length (AL), and 6) total body length (T). Width measurements of each respective body part were taken at the widest point. Length measurements of the thorax and abdomen were taken from the anterior end of the protergum to the caudal end of the first abdominal tergum (T_1-IT; T_1 includes the scutum and scutellum) and the anterior end of the second abdominal tergum to the caudal end of seventh abdominal tergum (IIT-VIIT) not including the stinger, respectively. All size measurements were taken in duplicate and averaged creating a single observation.

2.4. Fat body quantification

Ether extraction was performed to estimate the weight and relative size of the fat body of bees according to Wilson-Rich et al. (2008). Briefly, the abdomen of each bee was severed using surgical scissors and placed into separate holding cups to dry at 25°C for 3 days. Next, abdomens were placed in individual 1.5 ml microcentrifuge tubes to which 500 µl of diethyl ether was added. Abdomens were removed after 24 h and dried again for 3 days (same conditions). A Binder ED 53 drying oven was used to insure continuity of drying conditions over the duration of the study. Dried abdomens were weighed before and after ether extraction using a ORMA BCA200 electric laboratory balance with a precision 0.0001 g. The fat body weight (FBW) was calculated as the difference between the weight of each abdomen before and after washing with diethyl ether. The relative size of the fat body (FB%) was calculated as the proportional weight of the fat body relative to the weight of the dried abdomens prior to ether extraction (Wilson-Rich et al., 2008; Strachecka et al., 2021).

2.5. Data analysis

All procedures were carried out using a software package (Minitab statistical software package, Minitab©, New York, NY, USA). Statistical significance was set at *p*-value <0.05 and Tukey test was used for the *post hoc* pairwise comparison of means.

2.5.1. Worker bee type

Analysis of variance (ANOVA) was performed to detect significant differences in monitored metrics between the two types of sampled worker bees. A balanced linear model with interaction was used as follows:

$$y_{a,b,c,\ldots,k} = \mu + W_{a,b} + H_{j,k} + W * H + \varepsilon$$

where ***y*** is the dependent variable (*n* = 9; Weight, HW, TW, TL, AW, AL, T, FBW, FB%), **μ** is the overall mean, **W** is the fixed factor representing worker type (2 levels; Forager, Nurse), **H** is the fixed factor of hive (*n* = 5; *H1*, *H2*, *H3*, *H4*, *H5*), **W*H** is the interaction term, and **ε** is the random error.

Further analysis for any dependent variable significantly affected by **W** was conducted for forager and nurse bees separately. Unaffected variables were analysed using the whole dataset.

2.5.2. Effect of sampling date

Analysis of variance (ANOVA) was performed to detect significant differences in monitored metrics between the sampling months. A balanced linear model with interaction was used as follows:

$$y_{a,b,c,\ldots,k} = \mu + M_{a,b} + H_{j,k} + M * H + \varepsilon$$

where ***y*** is the dependent variable (*n* = 9; Weight, HW, TW, TL, AW, AL, T, FBW, FB%), **μ** is the overall mean, **M** is the fixed factor of sampling month (*n* = 12; January-December), **H** is the fixed factor of hive (*n* = 5; *H1*, *H2*, *H3*, *H4*, *H5*), **M*H** is the interaction term, and **ε** is the random error.

If a significant effect of **H** was found, the dataset was split accordingly and the effect of **M** analysed separately.

2.5.3. Effect of environmental factors and flower diversity

Analysis of variance (ANOVA) was performed to detect any changes in dependent variables of sampled bees according to environmental factors and flower diversity. All data were analysed following a general linear model procedure with interaction as follows:

$$y_{a,b,c,\ldots,k} = \mu + T_{a,b} + P_{c,d} + O_{e,f} + F_{g,h} + H_{i,j} + T * H + P * H + O * H + F * H + \varepsilon$$

where ***y*** is the dependent variable (*n* = 9; Weight, HW, TW, TL, AW, AL, T, FBW, FB%), **μ** is the overall mean, **T** is the fixed factor of monthly average environmental temperature (monthly average ambient temperature was divided into 4 levels; *Ta*, *Tb*, *Tc*, *Td*; range: 10-15, >15-20, >20-25, >25 °C; *see* Table 1), **P** is the fixed factor representing mean monthly precipitation (mean monthly precipitation was divided into 3 levels; *Pa*, *Pb*, *Pc*; range: 0-50, >50-100, >100 mm; *see* Table 1), **O** is the fixed factor representing monthly average daylength (monthly average daylength was divided into 4 levels; *Oa*, *Ob*, *Oc*, *Od*; range: 550-650, >650-750, >750-850, >950 h of daylight; *see* Table 1), **F** is the fixed factor effect of the monthly flower diversity (monthly flower diversity was dived into 3 levels; *Fa*, *Fb*, *Fc*; range: <5, 5-10, >10 species of flowering plants *see* Table 2), **H** is the fixed factor of hive ($n = 5$; *H1*, *H2*, *H3*, *H4*, *H5*), **T*H** is the interaction term between temperature and hive, **P*H** between precipitation and hive, **O*H** between daylight and hive, **F*H** between plant diversity and hive, and **ε** is the random error.

If a significant effect of **H** was found, the dataset was split accordingly and the effect of **T**/**P**/**F**/**O** analysed separately.

2.5.4. Correlation analysis

Pearson test for the assessment of correlation between measured metrics was used (Weight, HW, TW, TL, AW, AL, T, FBW, FB%) and was performed on both worker bee types separately. A statistically significant correlation was deemed 1) weak: $\rho < 0.300$, 2) mild: $0.300 < \rho < 0.600$, or 3) strong: $0.600 < \rho < 1.000$. The nature of the correlation was defined as follows: $+\rho$ or $-\rho$: positively or negatively correlated.

3. Results

Sampled colonies benefited from feed resource availability during the whole duration of the study. The diversity of flowering plants steeply increased during spring before peaking in early summer. This peak was followed by a drastic decrease over the course of the summer, bottoming in August. Summer dearth was followed by a mild restoration in fall. Limited flower diversity was noted in early winter which increased in February marking the onset of the foraging season. The various species of flowering plants encountered during the study period are reported per sampling month in Table 2.

Table 2. Summarising table of the monthly diversity of flowering plants in the honey bee flight area over the study period (February 2022-January 2023) in Sassari (Italy).

	Month											
	Jan*	***Feb***	***mar***	***Apr***	***May***	***Jun***	***Jul***	***Aug***	***Sep***	***Oct***	***Nov***	***Dec***
Species												
Acacia dealbata		X	X									
Anthemis arvensis			X	X	X							
Asphodelus ramosus		X	X									
Bellis perennis			X	X	X				X	X		
Borago officinalis				X	X	X						
Calendula arvensis		X	X	X	X	X			X	X	X	X
Centaurea					X							
Chrysanthemum croronarium				X	X							
Convolvulus arvensis							X					
Crepis vesicaria		X	X	X	X	X	X		X	X	X	
Cynara cardunculus						X	X					
Dittrichia viscosa									X	X	X	
Echium plantagineum			X	X	X	X						
Eucalyptus sp.			X	X	X	X	X	X	X			
Foeniculum vulgare					X	X	X	X	X	X		
Fumaria officinalis				X	X	X						
Galactites tomentosus				X	X	X						
Geranium molle			X	X								
Glebionis coronaria				X	X							
Helminthotheca echioides sp.							X	X	X			
Hypochaeris achyrophorus			X	X	X							
Malva sylvestris				X	X							
Onopordum horridum					X	X	X					
Oxalis pes-caprae	X	X	X	X	X						X	X
Prunus amygdalus		X	X									
Rafanus sativus				X								
Raphanus raphanistrum					X	X						
Reichardia picroides				X	X	X	X		X	X	X	X
Salvia rosmarinus	X	X	X	X	X	X			X	X	X	
Senecio vulgaris					X							
Sinapis alba			X	X	X	X						
Trifolium nigrescens			X	X	X							
Count	2	7	15	20	23	14	8	3	9	7	6	3
Category*	***Fa***	***Fb***	***Fc***	***Fc***	***Fc***	***Fc***	***Fb***	***Fa***	***Fb***	***Fb***	***Fb***	***Fa***

*Jan = January, Feb = February, Mar = March, Apr = April, Jun = June, Ju l= July, Aug = August, Sep = September, Oct = October, Nov = November, and Dec = December.

[**] Data for the month of January were collected in 2023 while the rest of the months regard the year 2022.

[***] Categories of environmental factors for statistical analysis: monthly flower diversity is dived into 3 levels; *Fa*, *Fb*, *Fc*; range: <5, 5-10, >10 species of flowering plants.

Mean values for all dependent variables for the whole database and per worker bee type are reported in Table 3.

Table 3. Mean morphological metrics of *Apis mellifera ligustica* nurse and forager bees recorded over a 12-month period (2022-2023).

	Overall[*]			***Nurse bee***			***Forager bee***		
	mean	***SD[**]***	***range***	***mean***	***SD[**]***	***range***	***mean***	***SD[**]***	***range***
Weight (g)	0.10	0.02	0.06-0.17	0.12	0.02	0.08-0.17	0.08	0.01	0.06-0.12
Head width (mm)	3.74	0.06	3.56-3.88	3.76	0.05	3.62-3.88	3.71	0.05	3.56-3.86
Thoracal width (mm)	3.77	0.05	3.56-3.93	3.77	0.05	3.56-3.92	3.77	0.05	3.58-3.93
Thoracal length (mm)	3.76	0.05	3.48-4.01	3.76	0.05	3.48-3.97	3.76	0.05	3.50-4.01
Abdominal width (mm)	4.27	0.15	3.82-4.79	4.35	0.14	4.00-4.79	4.18	0.11	3.82-4.50
Abdominal length (mm)	6.4	0.85	4.91-8.89	7.08	0.61	5.62-8.89	5.72	0.39	4.91-7.28
Total body length (mm)	11.84	0.79	10.21-14.43	12.42	0.66	10.41-14.43	11.27	0.38	10.21-12.78
Fat body weight (mg)	7.7	7.6	0-38.0	13.2	6.7	0-38.0	2.1	3.3	0.0-20.7
Fat body size (%)	29	20	0- 92	42	13	12-88	16	16	0- 92

[*] Overall mean values for the whole dataset (nurse and forager bees together).

[**] Standard Deviation.

A statistically significant difference (p <0.001) was found between worker honey bee types for all analysed metrics except for TW and TL ($F_{(1,1190)} = 2.11$, $p = 0.147$; $F_{(1,1190)} = 2.42$, $p = 0.120$). A significant effect of hive was found for TW, TL, and T ($F_{(1,1190)} = 13.30$, $p < 0.001$; $F_{(1,1190)} = 16.22$, p <0.001; $F_{(1,1190)} = 2.44$, $p = 0.045$). No interaction effect was detected between hive and worker type for any of the variables.

Post hoc analysis showed *H5* to be significantly different from other hives for TW and TL, while no decisive pattern was revealed for T. Mean TW and TL values for *H5* were lower than those of other hives (TW: *H1* = 3.78, *H2* = 3.78, *H3* = 3.78, *H4* = 3.78, *H5* = 3.75; TL: *H1* = 3.77, *H2* = 3.76, *H3* = 3.77, *H4* = 3.77, *H5* = 3.74).

No significant difference in TW and TL was found over the months for any of the hives.

Results of the analysis of variance on the effect of hive and sampling month for nurse and forager bees are reported in Table 4.

Table 4. Results of the analysis of variance for the effect of hive and sampling month on various metrics of *Apis mellifera ligustica* nurse and forager honey bees.

	Effect of hive			***Effect of Month***			***Interaction***		
	***p-value*[a]**	***F-value***	***df*[*]**	***p-value*[a]**	***F-value***	***df*[*]**	***p-value*[a]**	***F-value***	***df*[*]**
Nurse bees									
Weight	0.016	3.09	(4,540)	<0.001	50.02	(11,540)	0.220	0.64	(44,540)
Head width	0.637	0.64	(4,540)	<0.001	12.91	(11,540)	0.999	0.64	(44,540)
Abdominal width	0.299	1.23	(4,540)	<0.001	23.99	(11,540)	0.795	0.82	(44,540)
Abdominal length	0.213	1.46	(4,540)	<0.001	19.07	(11,540)	0.439	1.02	(44,540)
Total body length	0.009	3.42	(4,540)	<0.001	26.47	(11,540)	0.338	1.08	(44,540)
Fat body weight	0.602	0.69	(4,540)	<0.001	38.51	(11,540)	0.076	1.34	(44,540)
Proportional fat body size	0.744	0.49	(4,540)	<0.001	31.22	(11,540)	0.100	1.3	(44,540)
Forager bees									
Weight	0.568	0.74	(4,540)	<0.001	15.93	(11,540)	0.673	0.89	(44,540)
head width	0.483	0.87	(4,540)	<0.001	17.57	(11,540)	0.999	0.45	(44,540)
Abdominal width	0.846	0.35	(4,540)	<0.001	6.76	(11,540)	0.553	0.96	(44,540)
Abdominal length	0.023	2.85	(4,540)	<0.001	15.83	(11,540)	0.773	0.83	(44,540)
Total body length	0.401	1.01	(4,540)	<0.001	11.02	(11,540)	0.339	1.08	(44,540)
Fat body weight	0.709	0.54	(4,540)	<0.001	10.77	(11,540)	0.999	0.45	(44,540)
Proportional fat body size	0.621	0.66	(4,540)	<0.001	9.84	(11,540)	0.919	0.71	(44,540)

[a] Statistical significance set at p <0.005.

[*] Degrees of freedom.

Figure 1 and 2 show boxplots of the different variables significantly affected by month for nurse and forager bees respectively. The effect of month on the Weight and T of nurse bees according to hives is depicted in Figure 3 and 4. The effect of month on the AL of forager bees for the different hives is shown in Figure 5. Significantly different months are indicated by a red “*”. No significant interaction effect was found.

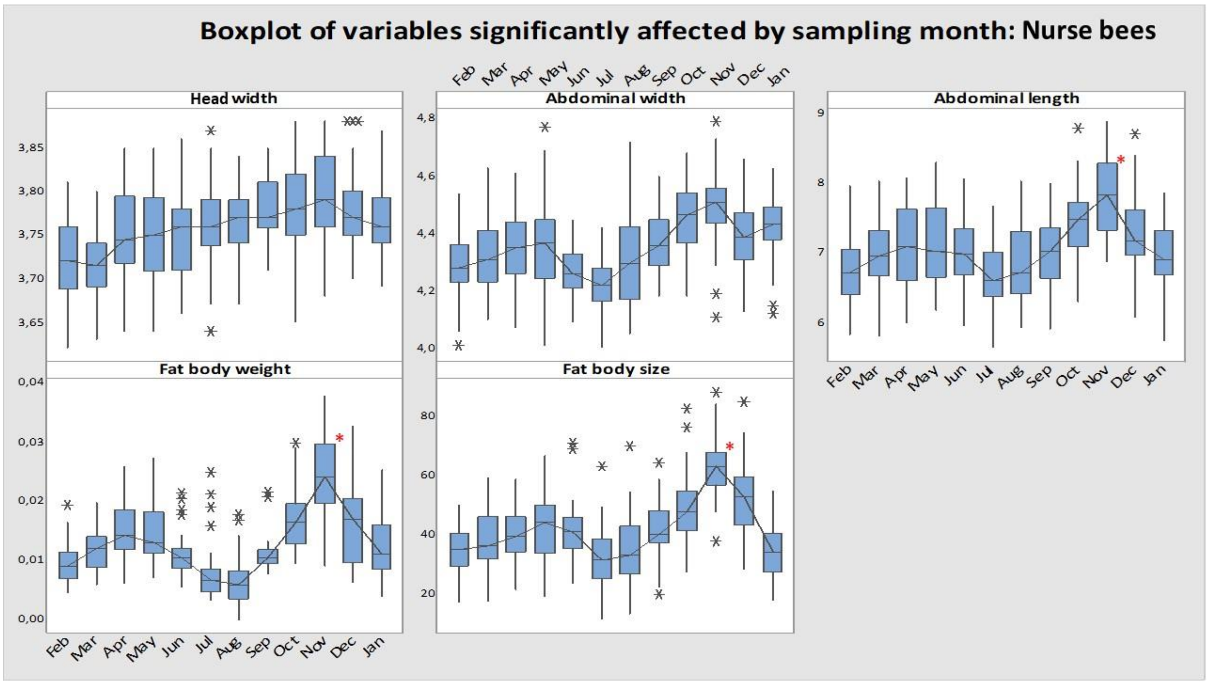

Figure 1. Box plots of Head width (mm), Abdominal width (mm), Abdominal length (mm), Fat body weight (mg), and Fat body size (%) of *Apis mellifera ligustica* nurse bees according to sampling months. The boxplot represents the interquartile range (IQR = Q3 − Q1) and bars represent first (Q1, top) and third quartiles (Q3, bottom) of metric values. Black "*" represent outliers and the black line represents the mean connect line. Red "*" indicates months significantly different from unmarked months. Feb = February, Mar = March, Apr = April, Jun = June, Jul = July, Aug = August, Sep = September, Oct = October, Nov = November, Dec = December, and Jan = January.

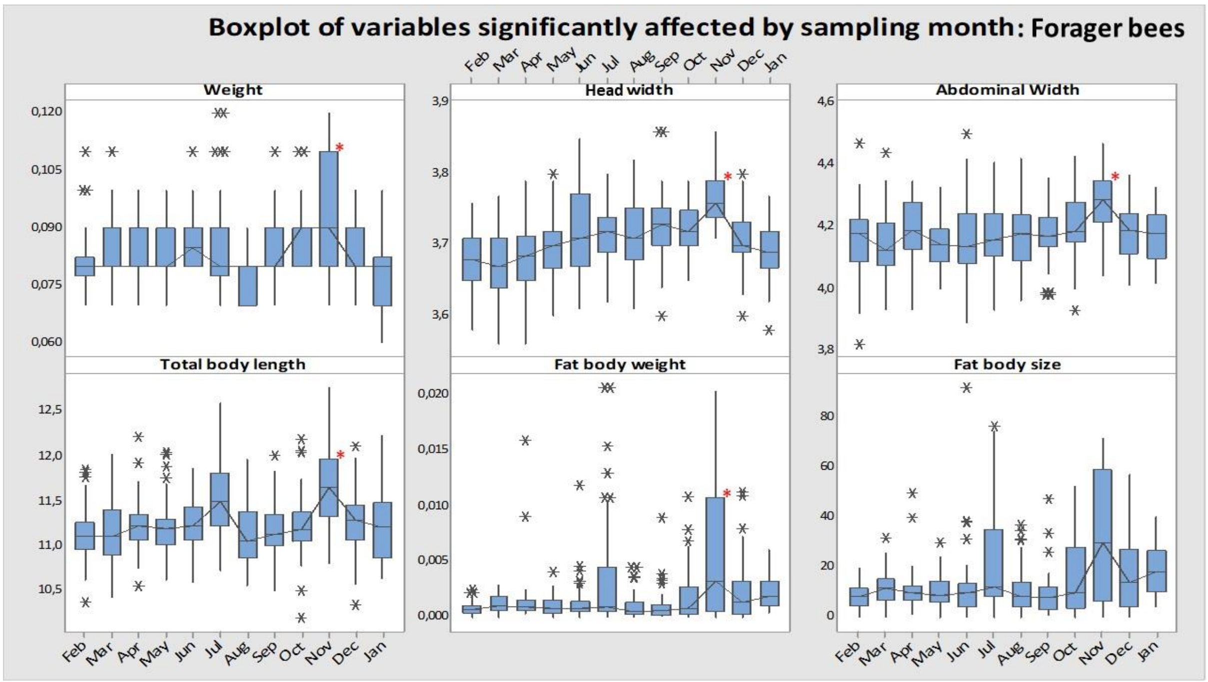

Figure 2. Box plots of Weight (g), Head width (mm), Abdominal width (mm), Total body length (mm), Fat body weight (mg), and fat body size (%) of *Apis mellifera ligustica* forager bees according to sampling months. The boxplot represents the interquartile range (IQR = Q3 − Q1) and bars represent first (Q1, top) and third quartiles (Q3, bottom) of metric values. Black "*" represent outliers and the black line represents the mean connect line. Red "*" indicates months significantly different from unmarked months. Feb = February, Mar = March, Apr = April, Jun = June, Jul = July, Aug = August, Sep = September, Oct = October, Nov = November, Dec = December, and Jan = January.

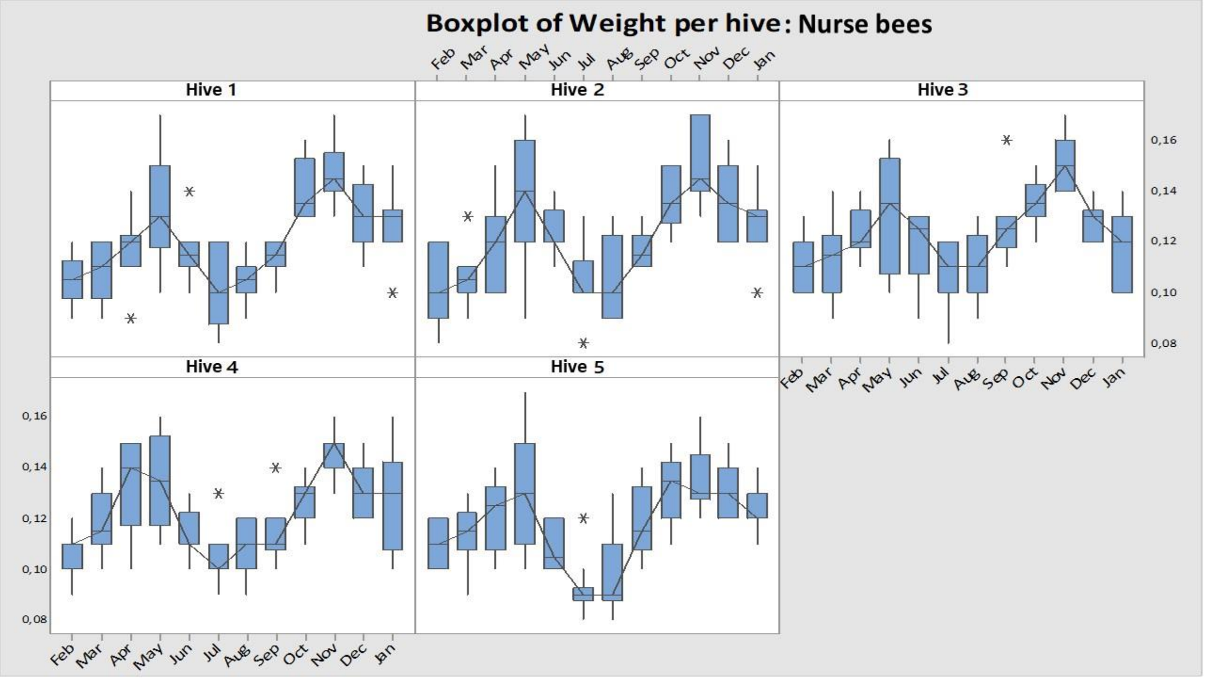

Figure 3. Box plots of the Weight (g) of *Apis mellifera ligustica* nurse bees according to hive and sampling months. The boxplot represents the interquartile range (IQR = Q3 − Q1) and bars represent first (Q1, top) and third quartiles (Q3, bottom) of metric values. Black "*" represent outliers and the black line represents the mean connect line. Feb = February, Mar = March, Apr = April, Jun = June, Jul = July, Aug = August, Sep = September, Oct = October, Nov = November, Dec = December, and Jan = January.

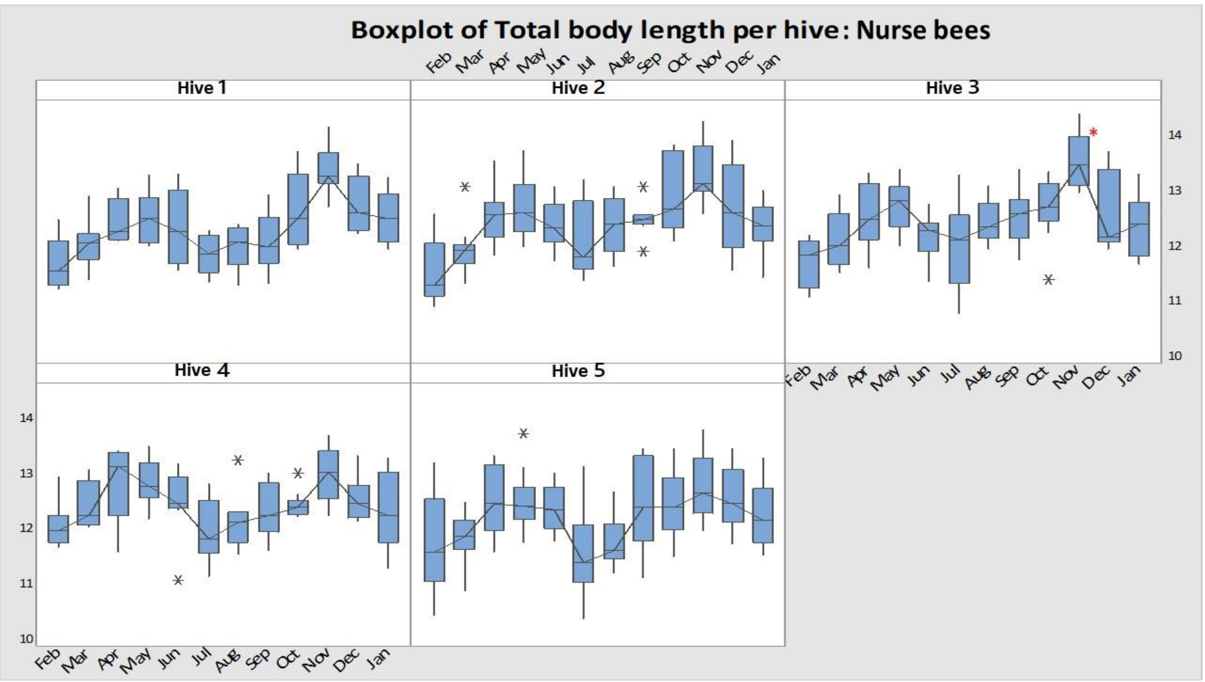

Figure 4. Box plots of the Total body length (mm) of *Apis mellifera ligustica* nurse bees according to hive and sampling months. Red “*” indicates months significantly different from unmarked months. The boxplot represents the interquartile range (IQR = Q3 − Q1) and bars represent first (Q1, top) and third quartiles (Q3, bottom) of metric values. Black “*” represent outliers and the black line represents the mean connect line. Red “*” indicates months significantly different from unmarked months. Feb = February, Mar = March, Apr = April, Jun = June, Jul = July, Aug = August, Sep = September, Oct = October, Nov = November, Dec = December, and Jan = January.

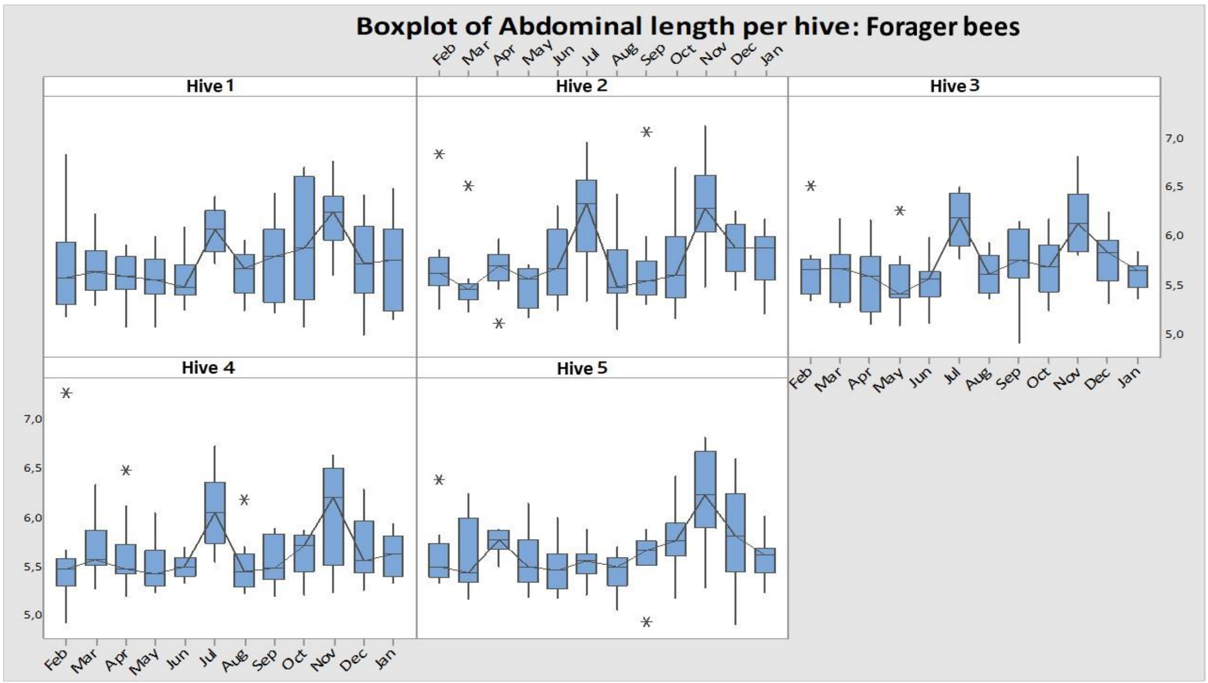

Figure 5. Box plots of the Abdominal length (mm) of *Apis mellifera ligustica* forager bees according to hive and sampling months. The boxplot represents the interquartile range (IQR = Q3 − Q1) and bars represent first (Q1, top) and third quartiles (Q3, bottom) of metric values. Black “*” represent outliers and the black line represents the mean connect line. Feb = February, Mar = March, Apr = April, Jun = June, Jul = July, Aug = August, Sep = September, Oct = October, Nov = November, Dec = December, and Jan = January.

Analysis of variance revealed no significant effect of environmental factors nor flower availability on TW for any of the hives. A significant effect of temperature ($F_{(3,\ 229)} = 2.66$, $p = 0.049$) and flower diversity ($F_{(3,\ 229)} = 3.44$, $p = 0.034$) on TL was found for *H3*, and a significant effect of temperature ($F_{(3,\ 229)} = 2.66$, $p = 0.049$) for *H2*. However, *post hoc* analysis showed no difference in TL between the groupings of various factors.

Results of the analysis of variance and *post hoc* analysis on the effect of environmental factors (mean monthly temperature, precipitation, daylength) and flower diversity for the dependent variables of nurse and forager bees are reported in Table 5. A significant effect of hive was found for Weight and T in nurse bees and are therefore reported here. Specifically, a significant effect of temperature was found on Weight and T for all hives (Weight: *H1*: $F_{(3,109)} = 23.47$, $p < 0.001$; *H2*: $F_{(3,109)} = 18.18$, $p < 0.001$; *H3*: $F_{(3,109)} = 13.61$, $p < 0.001$; *H4*: $F_{(3,109)} = 11.79$, $p < 0.001$; *H5*: $F_{(3,109)} = 11.79$, $p < 0.001$; T: *H1*: $F_{(3,109)} = 10.62$, $p < 0.001$; *H2*: $F_{(3,109)} = 10.28$, $p < 0.001$; *H3*: $F_{(3,109)} = 12.52$, $p < 0.001$; *H4*: $F_{(3,109)} = 3.93$, $p < 0.001$; *H5*: $F_{(3,109)} = 4.58$, $p = 0.005$). Weight was significantly affected by mean monthly precipitation for *H2* ($F_{(2,109)} = 5.11$, $p = 0.004$), *H3* ($F_{(2,109)} = 4.43$, $p = 0.014$), *H4* ($F_{(2,109)} = 6.93$, $p = 0.001$), and *H5* ($F_{(2,109)} = 5.11$, $p = 0.026$). Lastly, precipitation had an effect on T for *H1* and *H2* ($F_{(2,109)} = 3.98$, $p = 0.022$; $F_{(2,109)} = 3.98$, $p = 0.008$) and plant diversity on Weight for *H1* ($F_{(2,109)} = 3.52$, $p = 0.033$). No interaction effect was found for any of the factors for both nurse and forager bees.

Table 5. Results of the analysis of variance for the effects of environmental factors (mean monthly temperature, precipitation, daylength, and flower diversity) on various metrics of *Apis mellifera ligustica* nurse and forager honey bees.

	Temperature				***Precipitation***				***Hours of daylight***				***Diversity of flowering plants***			
	F-value	***df***[*]	***p-value***[a]	***post hoc***[**]	***F-value***	***df***[*]	***p-value***[a]	***Post hoc***[**]	***F-value***	***df***[*]	***p-value***[a]	***post hoc***[**]	***F-value***	***df***[*]	***p-value***[a]	***post hoc***[**]
Nurse bee																
Head width	8.25	(3,589)	<0.001	*Tb,Tc,Td>Ta*	7.17	(2,589)	0.001	*Pc>Pb,Pa*	2.20	(3,589)	0.087	*X*	7.10	(2,589)	0.001	*Fa,Fb>Fc*
Abdominal width	28.41	(3,589)	<0.001	*Tb>Ta,Tc>Td*	5.20	(2,589)	0.006	*Pc>Pb>Pa*	8.83	(3,589)	<0.001	*Oa,Ob>Oc,Od*	3.01	(2,589)	0.05	*Fa,Fb>Fc*
abdominal length	29.45	(3,589)	<0.001	*Tb>Ta,Tc>Td*	10.22	(2,589)	<0.001	*Pc>Pb,Pa*	3.41	(3,589)	0.017	*Oa,Ob,Oc,Od*	1.70	(2,589)	0.183	X
Fat body weight	39.14	(3,589)	<0.001	*Tb>Ta>Tc>Td*	18.82	(2,589)	<0.001	*Pc>Pb>Pa*	3.29	(3,589)	0.021	*Oa>Ob,Oc,Od*	2.21	(2,589)	0.111	*Fa,Fb,Fc*
Fat body size	40.36	(3,589)	<0.001	*Tb>Ta,Tc>Td*	39.31	(2,589)	<0.001	*Pc>Pb,Pa*	3.67	(3,589)	0.012	*Oa,Ob>Oc,Od*	2.26	(2,589)	0.105	*Fa,Fb,Fc*
Forager bee																
Weight	9.14	(3,589)	<0.001	*Tb>Tc>Ta>Td*	7.8	(2,589)	<0.001	*Pc>Pb,Pa*	1.3	(3,589)	0.272	*X*	6.9	(2,589)	0.001	Fb>Fc>Fa
Head width	18.2	(3,589)	<0.001	X	5.84	(2,589)	0.003	*Pc>Pa>Pb*	3.56	(3,589)	0.014	*Oa,Ob,Oc,Od*	0.65	(2,589)	0.520	X
Abdominal width	5.84	(3,589)	0.001	X	1.44	(2,589)	0.239	X	4.1	(3,589)	0.007	*Oa,Ob,Oc,Od*	1.93	(2,589)	0.146	X
abdominal length	10.57	(3,589)	<0.001	*Tb,Td>Ta,Tc*	9.83	(2,589)	<0.001	*Pc>Pa>Pb*	5.48	(3,589)	0.001	*Oa>Ob,Oc,Od*	14.16	(2,589)	< 0.001	*Fb>Fa,Fc*
total body length	8.09	(3,589)	<0.001	*Tb,Td,Ta,Tc*	7.82	(2,589)	<0.001	*Pc>Pb,Pa*	7.36	(3,589)	<0.001	*Oa,Od>Ob,Oc*	9.69	(2,589)	< 0.001	*Fb>Fa,Fc*
Fat body weight	9.07	(3,589)	<0.001	*Tb,Td,Ta,Tc*	6.34	(2,589)	0.002	*Pc>Pb,Pa*	6.56	(3,589)	<0.001	*Oa,Ob,Oc,Od*	6.44	(2,589)	0.002	*Fb>Fa,Fc*
Fat body size	8.28	(3,589)	<0.001	*Tb,Td,Ta,Tc*	4.01	(2,589)	0.019	*Pc>Pb,Pa*	5.85	(3,589)	0.001	*Oa,Ob,Oc,Od*	2.75	(2,589)	0.065	X

[a] Statistical significance set at $p < 0.005$.

[*] Degrees of freedom.

[**] Categories that were shown to be different through *post hoc* analysis (Tukey test) are separated by “>”, while groupings that are not different from each other are separated by “,”.

Results of the correlation analysis between various metrics are reported for nurse and forager bees in Table 6.

Table 6. Results of Pearson correlation analysis between morphologic metrics of nurse and forager bees.

	Weight	*Head width*	*Thoracal width*	*Thoracal length*	*Abdominal width*	*Abdominal length*	*Total body length*	*Fat body weight*
Nurse bees								
Head width	0.578**	/	/	/	/	/	/	/
P-value	<0.001	/	/	/	/	/	/	/
Thoracal width	0.241*	0.224*	/	/	/	/	/	/
P-value	<0.001	<0.001	/	/	/	/	/	/
Thoracal length	0.272*	0.216*	0.557**	/	/	/	/	/
P-value	<0.001	<0.001	<0.001	/	/	/	/	/
Abdominal width	0.638***	0.457**	0.323**	0.330**	/	/	/	/
P-value	<0.001	<0.001	<0.001	<0.001	/	/	/	/
Abdominal length	0.796***	0.768***	0.205*	0.244*	0.572**	/	/	/
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	/	/	/
Total body length	0.770***	0.636***	0.213*	0.259*	0.529**	0.794***	/	/
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	/	/
Fat body weight	0.753***	0.424**	0.104*	0.144*	0.493**	0.675***	0.666***	/
P-value	<0.001	<0.001	0.024	0.002	<0.001	<0.001	<0.001	/
Fat body size	0.310**	0.133*	0.045	0.069	0.183*	0.257*	0.293*	0.498**
P-value	<0.001	0.004	0.324	0.131	<0.001	<0.001	<0.001	<0.001
Forager bees								
Head width	0.214*	/	/	/	/	/	/	/
P-value	<0.001	/	/	/	/	/	/	/
Thoracal width	0.117*	0.780***	/	/	/	/	/	/
P-value	<0.001	<0.001	/	/	/	/	/	/
Thoracal length	0.072	0.692***	0.830***	/	/	/	/	/
P-value	0.079	<0.001	<0.001	/	/	/	/	/
Abdominal width	0.614***	0.317**	0.286*	0.219*	/	/	/	/
P-value	<0.001	<0.001	<0.001	<0.001	/	/	/	/
Abdominal length	0.731***	0.212*	0.095*	0.035	0.639***	/	/	/
P-value	<0.001	<0.001	0.020	0.396	< 0.001	/	/	/
Total body length	0.735***	0.218*	0.102*	0.063	0.636***	0.871***	/	/
P-value	<0.001	<0.001	0.012	0.125	<0.001	<0.001	/	/
Fat body weight	0.669***	0.204*	0.088	0.064	0.471**	0.649***	0.643***	/
P-value	<0.001	<0.001	0.053	0.160	<0.001	<0.001	<0.001	/
Fat body size	0.662***	0.212*	0.097*	0.071	0.458**	0.645***	0.652***	0.907***
P-value	<0.001	<0.001	0.034	0.121	<0.001	<0.001	<0.001	<0.001

*indicates a weak correlation.

**indicates a mild correlation.

***indicates a strong correlation.

4. Discussion

Nutrition is a key aspect influencing honey bee health and overwintering success (Brodschneider and Crailsheim, 2010; Steinhauer et al., 2018; Branchiccela et al., 2019; Bocquet et al., 2022). Nevertheless, there are relatively few studies that explore honey bee seasonal activity in southern temperate climates (Ricigliano et al., 2018; Maes et al., 2021). In this research, the nutritional status of the Italian bee (*A. m. ligustica*), a subspecies well adapted to the warm temperate climate of the Mediterranean, was studied (Ruttner, 1988; Gupta, 2014; Kovac et al., 2014). Specifically, individual weight, fat body, and size measurements (head, thorax, abdomen, and total body) were recorded on a monthly basis in order to detect temporal changes in the nutrient storage of worker bees during a complete annual cycle (2022-2023). Recorded parameters were analysed according to climatological factors and the availability of feed recourses (flower diversity) in order to get a better understanding of the annual bimodal dynamics of the honey bee workforce in a southern temperate Mediterranean climate.

Besides following seasonal variations in honey bee nutrition, novel data regarding two distinct worker bee types with varying biological age; in-hive (nurse bees) *Vs*. out-hive (forager bees) is presented. Given the consistent and fundamental behavioural, physiological, and nutritional differences between these two worker bee types (Amdam and Omholt, 2002; Toth et al., 2005; Toth and Robinson, 2005; Wilson-Rich et al., 2008; Ament et al., 2010; 2011; Alaux et al., 2018; Harwood and Amdam, 2021; Sarioglu-Bozkurt et al., 2022) authors hypothesised nutrition-related size metrics to vary significantly between them. Furthermore, as nurse and forager bees have different responses to similar conditions (Ament et al., 2010), the analysis of fixed factors (sampling time, environmental factors, and feed resource availability) was conducted separately for both cohorts.

While body size is a known indicator of nutritional stress reflecting the quantity and quality of food available during development in honey bees (Kunert and Crailsheim, 1988; Hoover et al., 2006; Scofield et al., 2015; Wang et al., 2016; Retschnig et al., 2021; Schilcher et al., 2022), to the best of our knowledge, no empirical evidence has so far been produced showing size variations between worker honey bees to be related to age division of labour. In fact, body size variations of worker bees within a single *A. mellifera* colony are believed to be negligible (Kerr and Hebling, 1964; Roulston and Cane, 2000; Chole et al., 2019). Here we show significant differences in nutrition-related size measurements between individual forager and nurse bees. With the exception of thoracal dimensions, all measured metrics differed between both worker bee types. Correspondingly, individual size measurements were strongly or mildly positively correlated to known biological markers of honey bee nutrition [body and fat body weight (Wilson-Rich et al., 2008; Smart et al., 2016; López-Uribe et al., 2020; Koubová et al., 2021; Retschnig et al., 2021)] (Table 6). These findings are in accordance with worker physiology, showing nurse bees to have substantially larger nutrient stores as compared to foragers (Amdam and Omholt, 2002; Toth et al., 2005; Toth and Robinson, 2005; Wilson-Rich et al., 2008; Ament et al., 2010; Alaux et al., 2018; Harwood and Amdam, 2021; Sarioglu-Bozkurt et al., 2022). The weak correlation between Weight and FBW *Vs.* HW in foragers is explained by the fact that these bees have hypotrophied hypopharyngeal glands (Amdam and Omholt, 2002; Döke et al., 2015). In contrast, these glands, which serve for the production of brood food, are well-developed in nurse bees (Fluri et al., 1982; Crailsheim, 1990; Amdam and Omholt, 2002; Döke et al., 2015; Ali et al., 2019). Since brood food is produced from Vg (Amdam et al., 2003; Seehuus et al., 2007), it is logical HW to be correlated to nutritional markers in this cohort.

The long-term monitoring of selected metrics allowed us to paint a detailed picture of the annual cycle of Italian bees in the study area from a nutritional point of view. In accordance with the seasonal adaptations of worker honey bees (summer *Vs.* winter bees) (Döke et al., 2015; Kunc et al., 2019; Yamada et al., 2019; Knoll et al., 2020; Koubová et al., 2021; Maes et al., 2021), a functional bimodal division of the honey bee cycle is followed.

The “summer-bee portion” of the nutritional cycle, running from mid-winter (end of December) to early fall (September) in this study, closely followed the nectar flow. When feed resources were abundant, individual honey bee nutrition increased and the opposite was seen during resource dearth (Kunert and Crailsheim, 1988; Ricigliano et al., 2018; 2019; 2021; Dolezal et al., 2019). Contrarily, a general increase in HW and TW was seen over the course of the foraging season. This corresponds to previous findings describing an increase in worker bee size within a yearly cycle (Ruttner, 1988; Sauthier et al., 2017).

Present data reflects a controversial increase in W, AL, T, FBW, and FB% of forager bees during the summer dearth period (peaking in July; Figure 2 and 5) which has been accredited to an explicit sampling error. Specifically, ambient temperatures at the time of sampling were so high that a large portion of bees had exited their hives and were found clustered around their respective hive entrances. This common strategy to prevent overheating (Abou-Shaara et al., 2017; Zhao et al., 2021) likely resulted in the sampling of a mixed population of worker bees rather than bees of a single biological age. Our deduction this finding to be a sampling error is supported by the overlapping ranges of measured metrics between both worker bee types for the month of July, as well as the increased variability seen for that sampling date.

Consistent with our present understanding of honey bee physiology in southern temperate climates of the northern hemisphere (Ruttner, 1988; Gupta, 2014; Ricigliano et al., 2018; Maes et al., 2021), the “winter-bee portion” of the nutritional cycle in this study was short and restricted to late fall/early winter (November-December). October can be considered a transition month as the shift from summer to winter bee-state is known to occur gradually within a colony and thus a balanced number of both castes is most likely present at this time (Mattila and Otis, 2007; Döke et al., 2015; Kunc et al., 2019). This portion of the honey bee year-cycle was characterized by a steep increase in nutrient storage in opposition to the overall diversity of feed resources (Table 2) with a subsequent decrease over the course of the winter period (Amdam and Omholt, 2002). This correlates well with current knowledge of honey bee seasonality with the arrival of winter bees primarily related to the disappearance of flowering plants in fall (Döke et al., 2015; Kunc et al., 2019; Knoll et al., 2020). As indicated by the significant difference in average monthly FBW, FB%, and AL (Figure 1), nutrient storage of nurse bees peaked in November, showing the presence of winter bees (Fluri and Bogdanov, 1987; Kunert and Crailsheim, 1988; Seehuus et al., 2007; Chen et al., 2016; Koubová et al., 2021). Whereas the overwintering state of honey bees in warmer climates differs from northern regions (e.g. sustained foraging and brood rearing activities), accumulation of fat and protein (Vg) is believed to be universal for overwintering honey bees in temperate zones (Ricigliano et al., 2018). Recent research monitoring Vg levels in the fat body of worker bees over a yearly cycle in the Czech Republic revealed a strikingly similar pattern even though nutrient storage in said research peaked in December (Koubová et al., 2021). Nevertheless, because sampling in the present study was conducted at the end of each month, nutrient storage of nurse bees could have peaked early to mid-December (as the noted brood rearing patterns would suggest) rather than in November.

The enlarged Weight, FBW, HW, AW, and T of forager bees in November, indicate that monitored hives exited their winter state somewhere between November and December. Indeed, increased morphological dimensions of forager bees during the winter dearth are indicative of a winter-bee-like state and can be considered remnants of the nutrient accumulation that occurred during in-hive activities (Knoll et al., 2022). Analogously, previous research has identified forager bees with increased morphological dimensions in early spring likely to be winter bees hatched the year before (Sauthier et al., 2017). Authors expected to see a delay in the detection of winter-bee-like foragers as compared to nurse bees. Nevertheless, the cessation of activities of Italian bees in this research was shorter than the sampling frequency likely resulting in the absence of a notable temporal divergence in seasonal transition between both worker bee types.

Overall changes in recorded metrics corresponded to the variation in environmental factors observed within the study period known to influence seasonal honey bee colony activity (Döke et al., 2015; Nürnberger et al., 2018; Kunc et al., 2019; Knoll et al., 2020; Koubová et al., 2021).

For both nurse and forager bees, monthly average ambient temperatures between 15-20 °C were correlated to the highest degree of nutrient storage (Table 5). These temperatures coincide with peak honey bee activity during the nectar flow in spring as well as the appearance of winter bees in fall. The fact that honey bees show two distinct physiological states within similar temperature ranges illustrates it is unlikely mean temperature alone influences the seasonal transition of honey bee colonies. Alternatively, interaction of temperature with other factors (e.g. photoperiod and feed resource availability), or the direction of temperature change in combination with reaching a threshold value could serve as a possible seasonal trigger (Mattila et al., 2001; Mattila and Otis, 2007; Döke et al., 2015; Nürnberger et al., 2018; Yamada et al., 2019).

Current understanding of honey bee behaviour in temperate climates describes the formation of a thermoregulating cluster when ambient temperatures drop below 10 °C (Döke et al., 2015; Cavoli et al., 2021). While average temperatures were well above this mark in November in the present research (and remained so for the whole duration of the study), minimum ambient temperatures did dip below 10 °C in November. More significantly, temperatures below this threshold were first recorded the month before (October). Given winter bees start appearing during this transition month, the first cold nights in fall could signal colonies to prepare for winter. The physiological mechanisms of how dropping ambient temperatures allow worker bees to accumulate Vg has previously been described (Huang and Robinson, 1995; 1996; Corona et al., 2007; Koubová et al., 2021).

In accordance with previous research efforts (Fluri and Bogdanov, 1987; Toth et al., 2005; Toth and Robinson, 2005; Koubová et al., 2021), an association between decreasing daylength and the accumulation of nutrients in the fat body of in-hive bees was noted. These results strengthen the hypothesis that decreasing photoperiod is involved in the seasonal appearance of winter bees (Döke et al., 2015; Nürnberger et al., 2018; Knoll et al., 2020) although present morphometrical results did not reveal further insights into the possible influence of daylength on the nutritional cycle of Italian honey bees.

Lastly, high average monthly precipitation (>100 mm) was consistently associated with an elevated nutrient status in both nurse and forager bees and coincided with the presence of winter bees. With the exception of AW in foragers, all measured metrics were significantly higher during months with high precipitation (Table 5). This finding is intriguing and could point towards weather conditions to be of particular importance in the seasonal dynamics of honey bees in southern temperate climates. Indeed, impaired meteorological conditions (“bad weather”) are known to influence honey bee demography by affecting the pheromone balance of a colony resulting in the active suppression of the biological maturation of young bees and the appearance of winter bees in fall (Huang and Robinson, 1992; 1996; Leoncini et al., 2004; Amdam et al., 2009; Döke et al., 2015; Knoll et al., 2020).

The flower diversity surveys conducted in the honey bee flight area provided a significant contribution to the study. We detected substantial variations in forage diversity (with specific regard to pollen availability) over the course of the monitoring period with an explicit pattern matching that of brood rearing. Because honey bee colonies do not store large amounts of pollen, the availability of this resource (the main nutrient supply for brood rearing) is strongly correlated to the brood rearing activity (Keller et al., 2005b; Mattila and Otis, 2007; Brodschneider and Crailsheim, 2010; Russell et al., 2013; Smart et al., 2016; Wood et al., 2018).

This pattern together with rest of the present data allowed us to identify two critical periods for honey bee health and nutrition in southern temperate climates, i.e. summer and winter dearth. While seasonal fluctuation in pollen availability showing one or two distinct peaks is not unusual (Ruttner, 1988; Keller et al., 2005b), large temporal variations in feed resource availability (nutritional irregularity) are known to affect honey bee health and longevity (Di Pasquale et al., 2013; 2016; Dolezal et al., 2019). Indeed, poor foraging conditions and related malnutrition are believed to be key factors in global colony losses (Naug, 2009; Brodschneider and Crailsheim, 2010; Steinhauer et al., 2018; Dolezal et al., 2019; Quinlan et al., 2023), especially in warm temperate climates (Maes et al., 2021).

High winter temperatures (Nürnberger et al., 2018) together with the prolonged availability of pollen (Mattila and Otis, 2007) offer a viable explanation for the late appearance of *diutinus* bees in this research as well as the sustained brood rearing observed for two out of the five hives (vanEngelsdorp et al., 2010; Maes et al., 2021). Although this might seem beneficial, continuous brood rearing during periods of limited pollen availability can cause premature exhaustion of fat and protein nutrient stores leaving colonies in a vulnerable state (Russell et al., 2013; Alaux et al., 2018; Nürnberger et al., 2018; Ricigliano et al., 2018; Maes et al., 2021). In effect, in-hive colony reserves and reserves within bees themselves are rapidly depleted in times of pollen dearth (Brodschneider and Crailsheim, 2010). Besides, flower diversity has been shown to be an important factor in honey bee nutrition since different pollen and nectar sources vary significantly in their nutritive value, e.g. protein and mineral contents (Keller et al., 2005a; Di Pasquale et al., 2013; Vaudo et al., 2015; Filipiak et al., 2017; Dolezal et al., 2019). Hence, even though nutritional recourses would be available during winter months, the limited variety of flowering plants during this time might not provide adequate nutrition in order to support brood rearing or honey bee colonies in general (Brodschneider and Crailsheim, 2010; Vaudo et al., 2015; Di Pasquale et al., 2016; Danner et al., 2017; Filipiak et al., 2017; Ricigliano et al., 2018; 2021; Branchiccela et al., 2019).

A prominent finding of the present study is that the nutritional state of *A. m. ligustica* workers was significantly negatively affected during periods of high ambient temperatures (>25 °C) and low precipitation (0-50 mm) (Table 5). With the exception of T and HW, all nurse metrics were lowest during the summer drought period (June-August) (Figure 1, 3, 4).

The precipitation pattern during the study period coincided with that of plant diversity during the "summer-bee portion" of the year which can be considered an illustration of the bottleneck effect of precipitation on plant growth in warm and dry Mediterranean climates (Peñuelas et al., 2004; Schweiger et al., 2010). The noted influence of weather on honey bee nutrition in summer therefore likely stems from an indirect effect on plants resulting in an overall resource dearth (Gordo and Sanz, 2005; Le Conte and Navajas, 2008; Hegland et al., 2009; Switanek et al., 2017; Kunc et al., 2019; Cavoli et al., 2021). Moreover, hot and dry conditions have been shown to reduce nectar and pollen production and the overall nutritional quality of these resources as well (Le Conte and Navajas, 2008; Schweiger et al., 2010; Cavoli et al., 2021). For these reasons, in addition to the winter dearth, summer food shortages could be of serious concern for honey bee colonies in southern temperate climates. This could be especially true in the face of accelerated climate change (Peñuelas et al., 2004; Le Conte and Navajas, 2008; De La Rúa et al., 2009; Russell et al., 2013; Flores et al., 2019; Stanimirović et al., 2019; Insolia et al., 2022; Kiraç and Birer, 2023) as conditions in the Mediterranean head towards a similar scenario seen in particularly arid climates such as in the Middle East (Carvalho et al., 2022; Kiraç and Birer, 2023) where summer droughts are a key factor in colony losses since many plants suffer from heat stress leading to feed shortage for honey bees (Hristov et al., 2021).

Lastly, the present data supports the observation that climate change (high ambient temperatures in particular) is causing a disproportionate shift in plant and honey bee phenology in the Mediterranean, resulting in temporal mismatches with possible nutritional consequences (Gordo and Sanz, 2005; 2006; Hegland et al., 2009; Nürnberger et al., 2018; Nath et al., 2022). Indeed, a particularly early initiation of the foraging season, well before the start of the nectar flow, was noted followed by a sharp decrease in nutrient storage over the course of winter (Figure 1).

5. Conclusion

The present research contributes to our understanding of the seasonal dynamics of honey bees in a southern temperate climate showing a short cessation of activities in late fall/early winter coupled with an increase in nutrient storage of in-hive bees. While the fall decrease in feed resources appears to be the main factor governing honey bee seasonality, a combination of changing environmental factors seems to be required for the arrival of winter bees. The continuous but limited availability of flowering plants and forgiving ambient temperatures during winter likely allowed for the observed brood rearing pattern and consequential sharp decrease in nutrient storage over the winter dearth period.

In addition, a first description of the annual nutritional honey bee cycle in a southern temperate climate is presented showing two critical timepoints. Overall, our results contradict the common assumption that warm climates are more suited for honey bees as besides winter, the Mediterranean summer, which is characterised by droughts and high temperatures, was identified as a second critical timepoint. It seems precipitation plays a particularly important role in southern latitudes, influencing nutrition in both the summer- and winter-bee portion of the honey bee year-cycle. Finally, present data support the notion that climate change is expected to have significant effects on honey bees in temperate Europe in the decades to come, especially through a pronounced impact on plants indirectly affecting health and nutrition.

Our results provide valuable insights into the seasonal and nutritional dynamics of locally adapted *A. m. ligustica* populations that could aid beekeepers to make management decisions in relation to environmental factors and availability of flowering plants with the ultimate goal of improving overwintering success and preventing unnecessary colony losses.

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DECLARATION OF COMPETING INTEREST

Authors declare that no conflict of interest exists.

AUTHOR CONTRIBUTION

Stephane Knoll: data collection and analysis, and paper writing; Valeria Fadda: data collection; Fahad Ahmed: manuscript review; Maria Grazia Cappai: project design, data analysis and manuscript review.

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CHAPTER 5

Conclusions

Honey bees are fascinating creatures who have shown great adaptability to a variety of conditions. The evolution of a seasonal cast system allowing these insects from (sub)tropical origin to colonize temperate climates with “extreme” seasonal variations is a great example of this. On the other hand, widespread global colony losses illustrate the fragility of *Apis mellifera* in an increasingly hostile environment. Indeed, these social insects are under growing threat of anthropogenic pressures, which include the spread of pests and diseases (*Varroa destructor* being the most well-known case), pesticide use, agricultural intensification, environmental contamination, and climate change. As humans have “modified” the honey bee’s environment, we have a responsibility to take the necessary actions to safeguard and conserve the species. Besides, this is in the best interest of all of us. Not only because humans increasingly depend on honey bees for sufficient agricultural production, but because these insects (and pollinators in general) ensure healthy ecosystems and thus are fundamental for most life on earth.

The overreaching goal of this dissertation is, in some small way, to contribute to the current knowledge of *Apis mellifera* with the hope of aiding in the fight against global honey bee colony losses. As winter represents a period of particular vulnerability for colonies in temperate climates of the northern hemisphere, and most colony losses occur during this time (although summer losses are an increasing concern), this dissertation starts off by centralising current knowledge on honey bee seasonality (summer *Vs.* winter bees). A detailed account of the central workings of juvenile hormone and vitellogenin is given, illustrating how honey bees have adapted molecular pathways related to insect reproduction and development into an effective physiological system with dual functionality across temporal patterns. In the short term, this system regulates the age division of labour among sterile female workers ensuring proper colony performance in times when ample resources are available. Conversely, when favourable conditions are lost, this system governs the seasonal appearance of *diutinus* bees who safeguard colony survival in winter and kickstart the next honey bee year-cycle. The fundamental role of nutrition in honey bee seasonality is exemplified by vitellogenin (the main nutritional storage molecule in honey bees produced and stored by the fat body) accounting for the typical features associated with winter bees including longevity. Environmental factors, feed resource availability, brood rearing, and colony demography have all been shown to have intertwining effects on honey bee seasonal dynamics.

Even though extensive research efforts have allowed for a rather comprehensive understanding of these mechanisms, some research gaps remain. Among these is the lack of knowledge of honey bee seasonality and overwintering in southern temperate climates. Given nutrition (with vitellogenin levels of individual bees synonymous with their nutritional status) underlays the profound physiological differences between the two seasonal worker bee casts, the long-term monitoring of worker bees in a southern temperate climate (Sassari, Italy) based on markers of honey bee nutrition was considered in order to address this issue. Since such markers are generally scarce, novel easy-to-use markers were first conceived and evaluated. In this regard, robust measurements of individual size (head width, thoracal width and length, abdominal width and length, and total body length) were hypothesized to be useful markers of individual honey bee nutrition.

The preliminary evaluation of selected size metrics performed on foragers revealed abdominal and total body length to be suitable candidate markers as they were well correlated to previously established markers of individual honey bee health and nutrition (body weight, fat body weight, and proportional fat body size). Furthermore, selected metrics were successfully employed for the detection of winter-bee-like foragers during a 7-month monitoring effort (2019-2020). In this "proof of concept", abdominal length and head width showed the most promise as a peak in these metrics in winter indicated the presence of bees with increased nutrient storage likely to be *diutinus* bees exiting their winter state.

With this information in hand, nutritional monitoring over the course of a whole year was conducted (2022-2023) and the data analysed according to external factors known to be (at least partially) involved in the seasonal dynamics of *Apis mellifera* in temperate climates; temperature, precipitation, daylength, and flower diversity. In accordance with current knowledge of honey bee seasonality, the fall decrease in feed resources appeared to be the main factor governing the appearance of winter bees with rather secondary effects of temperature and daylength. Precipitation was found to have a significant effect on honey bee dynamics and could be a factor of particular seasonal importance in southern temperate climates.

Contrary to northern latitudes, the overwintering state of honey bees in the study area was short as was illustrated by the simultaneous peak in nutrient storage of both nurse and forager bees within the same month. This being said, a brood stop of approximately two weeks (for 3/5 hives) was noted which was shorter than the sampling frequency suggesting this might have led to the distinct appearance of these worker types to be imperceptible. Interestingly, while the duration of the winter-bee-state in both monitoring efforts reported in this dissertation was identical, they occurred in distinct months showing temporal plasticity of the mechanism behind honey bee seasonality. While the relationships with regard to external factors were not investigated in the first monitoring of forager bees, it is likely variations in climatological factors and feed availability from year to year resulted in this discrepancy.

Besides exploring seasonal dynamics, long-term monitoring of selected size measurements resulted in a detailed description of the nutritional year-cycle of honey bees in a southern temperate climate. This generated valuable insight that could guide beekeepers to take management decisions in relation to environmental factors and the availability of flowering plants in order to optimise the health and nutrition of their colonies. Fluctuations in nutrition-related metrics in correspondence to changes in flower diversity allowed for the identification of two critical periods for honey bee nutrition in the study area; summer and winter dearth. While both dearth periods have similar consequences for honey bees, they occur for different reasons. 1) Winter dearth was characterized by low temperatures and high precipitation: in this scenario, relatively high winter temperatures likely result in phenological advancement of both plants and honey bees which ultimately leads to malnutrition. Specifically, the relatively high ambient temperatures cause a limited amount of plants to flower in winter, which (in combination with high temperatures) stimulates bees to remain active. Nevertheless, the limited feed availability puts excessive nutritive pressure on honey bee colonies, resulting in exhaustion towards spring. 2) Summer dearth was characterized by high temperatures and extensive droughts (extremely low precipitation): these conditions commonly lead to heat stress for plants causing feed shortages for bees in times of peak activity, again resulting in excessive nutritive pressure for colonies.

Both these scenarios were considered the result of (or are at least exacerbated by) rapid global warming the world is facing today demonstrating how climate change could pose an increasing threat to honey bees in the Mediterranean if the continuation of this trend is manifested. Since the Mediterranean is regarded as the “forefront” for climate change in Europe, insights gathered here could provide a context for extrapolation on how honey bees in colder temperate climates may be affected in the future. Moreover, as climate change might favour the survival of honey bee subspecies adapted to specific climatic conditions, the insights into the activity, seasonality, and nutrition of Italian bees (*A. m. ligustica*; a subspecies generally well adapted to the warm temperate climate of the Mediterranean) generated in the context of this dissertation could prove invaluable (e.g. as a range expansion northwards is expected).

Lastly, results of the long-time monitoring of individual size measurements in 2022-2023 were in accordance with those of 2019-2020. Since honey bees from two different apiaries were monitored, some size variations were expected. Nevertheless, size dynamics of forager bees showed a strikingly similar pattern with practically identical correlation results. This provides added power to the preliminary assessment of selected morphological metrics conducted on a single hive with a relatively low sample size. In addition, comparable results are presented for nurse bees showing abdominal length, abdominal width, and total body length to be candidate markers in this cohort as well.

All in all, the present dissertation reports on the potential of robust individual size measurements as markers for honey bee nutrition that could offer significant advantages in the context of research efforts and the monitoring of honey bees in general. In addition, insights into the seasonal dynamics and the year-round nutritional status of locally adapted *A. m. ligustica* are presented that can serve as a cornerstone for future honey bee research in southern temperate climates on these topics. Ideally, knowledge gained from this dissertation will be applied to improve overwintering success and prevent unnecessary honey bee colony losses.