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Integrated phenotypic and genetic assessment of performance traits in *Apis mellifera*

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The Pedigree of Honey
Does not concern the Bee -
A Clover, any time, to him
Is Aristocracy –

E. Dickinson

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Abstract

Honey bees play an important role in modern agriculture as farm animals and crop pollinators and represent an animal model in scientific research. Since few decades, managed honey bees are facing large scale losses worldwide due to the interaction of several biotic and abiotic factors such as the spread of pathogens and parasites, the habitat loss, the use of pesticides, and the occurrence of climate changes. For years, beekeepers have controlled deadly pathogens such as *Varroa destructor* with acaricides (pyrethroid), but widespread chemical resistance is manifesting. Alternative management strategies could be developed to characterize and select bees with heritable traits allowing them to resist mites and diseases. Hygienic behaviour (HB) is a heritable phenotype that confers colony level resistance against brood disease, with a potential effect on the damaging parasitic mite *V. destructor*. Unfortunately, breeding such bees is complicated as the assays involved to characterize such phenotype are time-consuming and expensive if performed on a consistent number of colonies. Additionally, the mechanisms behind social immunity are not yet fully understood. These issues have motivated the scientific community to develop research tools that can offer insight into the causes of declining bee health as well as identify biomarkers to guide breeding programs.

The studies presented in this thesis are the results of the collaboration between the Animal Genetic Group of the University of Milan and an Italian beekeeping company, which made available its animals for the majority of the studies presented in this work. The first two chapters of this thesis focus on three important aspects regarding hygienic behaviour: in-field phenotypic implementation of a method to measure HB on large testing populations, its genetic parameters estimation and developing molecular tools for the identification of potential HB genetic markers from breeding queens.

Queens are the only fertile females of the colony, responsible for laying eggs and maintaining the cohesion of the colony. The quality of the queen may have an important effect on a colony's development, productivity and survival and queen failure or loss is considered a cause of the decline of colonies worldwide. The queen quality, resulting from her genetic background, developmental condition, mating success and environment, can be assessed by some morphological measures. We investigated the genetic parameters of some traits that describe the quality of bee queens in the third chapter.

Beside queen morphology, in the fourth chapter, we investigated also the morphology of the worker bees with Computed Tomography (CT). In this study, a non-invasive CT technique and image analysis approach coupled with brood manual inspection was used to clarify the relationship between honey bee pupa length and its varroa mite infestation status, developmental status and spatial position within the brood comb. The results of this chapter suggest that the CT-scan may represent a suitable non-invasive tool to investigate the morphology and developing status of honey bee brood.

Finally, since honey yield is one of the major breeding goal a beekeeping company, we investigated the genetic parameters for honey yield in a small testing population of honey bees in Northern Italy both considering the total honey yield and the single harvests of a colony within a year on data collected over a period of three years.

Sintesi

Le api svolgono un ruolo importante nell'agricoltura moderna sia come animali da reddito che come impollinatori e possono essere un valido modello animale per la ricerca scientifica. Negli ultimi decenni, le api sono in declino in tutto il mondo a causa di diversi fattori biotici e abiotici connessi tra loro, quali la diffusione di agenti patogeni e parassiti, la perdita dell'habitat naturale, l'uso di pesticidi e il verificarsi di cambiamenti climatici. Per anni, gli apicoltori hanno contrastato l'acaro *Varroa destructor* attraverso l'uso di acaricidi (principalmente piretroidi), ma la resistenza a questi farmaci da parte dell'acaro limita considerevolmente la loro efficacia. Una soluzione potrebbe essere sviluppare strategie di gestione alternative per caratterizzare e selezionare api resistenti a parassiti e alle malattie della covata. Il comportamento igienico (dall'inglese Hygienic Behavior, HB) è un carattere ereditabile che conferisce a livello della colonia resistenza contro le malattie della covata e potenzialmente contrasta il dannoso acaro *V. destructor*. Purtroppo, selezionare le api per i caratteri che più interessano l'apicoltore presenta diverse peculiarità in quanto le analisi necessarie richiedono tempo e sono costose se eseguite su un numero consistente di colonie. Inoltre, i meccanismi alla base di questa immunità sociale non sono ancora del tutto chiari. Alla luce di ciò, la comunità scientifica è motivata a sviluppare strumenti di ricerca in grado di offrire informazioni sulle cause del declino della salute delle api e a identificare biomarcatori per guidare i programmi di selezione. La prima parte di questa tesi si è concentrata su tre aspetti importanti riguardanti il comportamento igienico: implementazione in campo di un metodo appositamente ottimizzato per misurare l'HB su popolazioni di test di grandi dimensioni, la stima dei parametri genetici per il comportamento igienico e lo sviluppo di metodologie molecolari per l'identificazione di potenziali marcatori genetici per l'HB misurati direttamente sulle regine.

La regina è l'unica femmina fertile della colonia, responsabile della deposizione delle uova e della coesione della colonia di api. La qualità della regina può avere un effetto importante sullo sviluppo, la produttività e la sopravvivenza di una colonia. Infatti regine con scarsa attitudine o che vengono a

mancare prematuramente sono considerate una tra le cause principali della perdita delle colonie in tutto il mondo. La qualità della regina, risultante dalla sua genetica, dalle condizioni di sviluppo, dal successo di accoppiamento e dall'ambiente, può essere valutata mediante alcune misure morfologiche. In questa tesi sono stati studiati i parametri genetici di alcuni tratti che descrivono la qualità delle regine delle api.

Oltre alla morfologia della regina, nel quarto capitolo abbiamo studiato anche la morfologia delle api operaie con la tomografia computerizzata (CT). In questo studio, la CT, l'analisi delle immagini accoppiati con l'ispezione manuale della covata sono stati usati per chiarire la relazione tra lunghezza della pupa dell'ape operaia e lo stato di infestazione da *V. destructor*, stato di sviluppo e posizione spaziale all'interno del favo. I risultati di questo capitolo suggeriscono che la CT può rappresentare uno strumento adatto e non invasivo per indagare la morfologia e lo stato di sviluppo della covata delle api.

Infine, abbiamo studiato i parametri genetici per la resa del miele in una piccola popolazione test nel Nord Italia considerando sia la produzione netta totale sia le singole produzioni di una colonia in un anno, stimando ereditabilità e ripetibilità.

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Thesis' outline

My research project aimed to find suitable and rational collection of phenotypes and to explore the analysis of various datasets to estimate genetic parameters like heritabilities. This thesis is characterized by a transversal approach: from in-field phenotypic data collection to SNP discovery. My work focussed on important and different phenotypes for beekeeping such as hygienic behaviour, quality of the queens, potential new approaches for in-vivo studies and honey production.

Thanks to the collaboration with an Italian beekeeping company (Melyos ssa) it was possible to carry out the studies on an acceptable (but still limiting) number of animals and thanks to Prof. Brascamp and Prof. Bijma I improved my knowledge in quantitative genetics and was supported by them for the genetic analysis in this thesis.

My thesis contains five chapters reporting various issues I studied in the context of phenotypic and genetic assessment of performance traits, preceded by an introduction setting the scene and followed by general conclusions.

The first chapter is about the development and application of a variant of the freeze killed test and to estimate genetic parameters for hygienic behaviour. Our aim was to develop a tool for professional beekeepers who perform large scale phenotyping. The application of such test on the breeding population of the beekeeping company made it possible to estimate heritability and repeatability for hygienic behaviour.

On the occasion of the performance testing, I collected the wings of the queens that were enrolled in the breeding population. The second chapter is on the molecular genetic research I carried out in the last part of my PhD when I could develop a method to extract DNA from queens' wings in order to explore genetic variability and potential association with the hygienic ability of the colonies. This chapter is a preliminary report on the DNA extraction method and sequence analyses that I carried out under the supervision of Prof.ssa Chessa.

The third chapter is a project that was commissioned by the beekeeping company to the University, given the volume of queens they produce and sell and the need to improve queens' quality. The aim was to explore any potential association between queens' morphology and reproductive traits. The chapter reports the first and preliminary genetic parameters of traits that could be used as proxies for queens' quality.

Chapter four is a project I personally developed, to investigate the possibility to visualize *Varroa destructor* by computed tomography (CT). It was not possible to visualize directly the mite, but I noticed that the length of the developing pupae was variable and looked promising to study. The chapter reports the results of the experiment we carried out on one brood comb.

Chapter five is a preliminary report for the analysis we carried out on honey yield. The improvement of honey production usually is one of the major breeding goals for a beekeeping company. Therefore, data were collected over a period of three years on the same breeding population enrolled for hygienic behaviour studies and queen quality. Besides the total yearly harvest, we chose to investigate the variability of separated honey productions within the productive season in order to test the repeatability of honey yield within a year.

General introduction

Apis mellifera is an insect bred for millennia by humans. Primordial forms of beekeeping in artificial hives date back to the ancient Egyptians, about 2500 B.C., who were used to transport the hives on boats along the Nile to follow blooming (Contessi, 2015).

The honey bee represents a highly relevant productive livestock not only for its products such honey, pollen, bee wax, propolis and venom, but also because together with wildlife pollinators it plays a key role for ecosystems and biodiversity, thanks to its generalist pollinator nature. Therefore, it could be considered the third most important economic livestock species, after cattle and swine (Tautz, 2008).

Admirable for the precise hierarchical social organization, for its communication and for the complex mechanisms that regulate its life, this remarkable insect was and still is intriguing many scientists.

Honey bees are social insects living in colonies of tens of thousands of individuals (from few thousands to tens of thousands, depending on the season). Colony members live in a single nest and work together creating, what is defined, a 'superorganism' (Wilson and Sober, 1989). Indeed, the colony is governed by a rigid eusocial structure and division of tasks. Bee societies do not derive from the gathering of individuals, but each individual is the offspring of the queen and sibling of each other. Therefore, very often the bee colony is called “colony”. Colonies live on bee wax frames usually built into natural caves or artificial boxes provided by men, the hive. The structural unit of the hive is the comb, which is made of wax secreted from small glands on the underside of the worker bee. The combs consist of a double set of horizontally hexagonal cells slightly inclined upwards, adjacent to each other and sharing the bottom, in a space saving design. The combs hang vertically and side by side. The space between the combs is not random but appositely built by the bees and it allows two bees to pass back to back. This space is known as the “bee space” and it is the cornerstone of moveable comb beekeeping. Two sizes of hexagonal cells exist those for the worker bees and those

for drones, the males, which are slightly larger. According to the need of a new queen for the colony, royal cells are built by worker bees, which are characterized by an acorn-shape and the downwards opening.

As holometabolous insects, honey bees undergo a four-stage process of development: egg, larva, pupa and adult. The young stages are known as brood and their development takes place inside the cells in the brood combs.

The colony is composed of three types of individuals. A fertile female, *e.g.* the queen, many thousands of sterile females, represented by worker bees, and few hundreds of males or drones, which are present only from spring to autumn (Winston, 1987; Contessi, 2015). The three types of individuals are distinguished easily by their morphology. The worker bees have the body covered with thick hairs, the wings of a length almost equal to that of the body and the eyes are well separated. The drones are larger, stockier, have longer wings and huge eyes that meet each other on the forehead. The queens are larger and tapered, with a swollen abdomen that protrudes from the wings' tip abundantly (Contessi, 2015).

Development and duties of the queen

The queen bee is the only fertile female of the colony and therefore, the mother of all the individuals of the hive. She develops from a fertilized egg, which is laid in the royal cell by her mother (another queen). Royal cells usually are built by worker bees along the borders of the combs. From the egg, after three days, a larva is born and grows for five to six days, undergoing 5 moults and feeding exclusively with royal jelly. Afterwards, worker bees seal the royal cell. Inside the sealed royal cell, the larva spins a silken cocoon and undergoes the last moult, becoming a pupa. The pupa takes seven days to become insect. The fully developed queen bee takes 15 to 17 days to hatch from the time the egg was laid. Generally, after 5 to 15 days from hatching, the queen gets ready to perform mating flights. The mating of queen bees takes place in particular sites, where drone and queens meet together

in the so-called “congregational area”. Mating occurs in flight. After mating, drones lose their reproductive organ and die. Otherwise, a queen bee can mate with many drones. If adverse conditions do not permit the fecundation of the queen (*e.g.* bad weather, no drones availability) the queen will become a drone laying one, as she will lay only unfertilized haploid eggs. A colony with a drone laying queen is destined to die if the beekeeper does not intervene promptly. If the mating was successful, the fertilized queen will return to her natal hive and begin to lay eggs after 2 to 5 days. The semen in the spermatheca usually lasts four to five years. Therefore, her main duty inside the colony is to lay eggs. The fertilization of eggs does not occur right after mating, but only at the time of oviposition. Indeed, after mating, queens keep seminal fluid in a special structure called spermatheca, where the semen is maintained vital for the entire lifespan of the queen (up to five years). The opening and closing of the spermatheca are controlled by a special valve. Most likely the size of the cell establishes a reflex arc that regulates the opening of the valve of the spermatheca. In the presence of a small cell, the valve opens, releasing enough sperm for the fertilization of the egg, from which a female will be born. Otherwise, in the presence of a larger cell, the valve is kept closed and the queen lays a haploid egg, from which a male will be born. However, is not the queen who decides whether to give birth to females or males, but any decision is up to the workers, which, depending on the size of the cells that they build, will determine the sex of the individuals who will be born (Contessi, 2015).

Naturally, wild life honey bees replace their queen before she becomes sterile, by raising new queens from fertilized eggs.

Development and duties of drones

Males are produced at the end of winter and they are generally reared by the colony when young queens are likely to be present, as their main purpose is to fertilize the virgin queens. Drones develop from unfertilized eggs laid by the queen in slightly bigger cells than those of worker brood. Drones are larger than workers and their development takes longer. The egg hatches after 3 days and it will

be fed initially with royal jelly, and after about three days the diet changes and will be based on honey, pollen, and water. After six days the drone cell is sealed by worker bees with a domed cap of wax. In the sealed cell the larva undergoes 5 moults, spins a silken cocoon and finally metamorphosis occurs. The drones emerge after 24-25 days. Then, after a week of being fed and helping themselves to honey stores, they are ready to fly. After 12-20 days from the birth they are able to mate, but they reach fully sexual maturation usually after 30-40 days. Their life usually last about 50 days. They are extremely strong flyers and have large eyes and highly developed vision, as the mating with virgin queens occur in flight. On warm still days they assemble in small clouds in places known as drone congregational areas waiting for queens. At the end of the summer the drones are left out from the hive.

Development and duties of workers

Worker honey bees are born from fertilized eggs. The larva hatch after three days from the oviposition. For the first three days the bee larva is fed by the workers exclusively with royal jelly, afterward the diet is composed of honey, pollen, and water. The larva undergoes 5 moults in the sealed cell. The fully developed insect is born after about 21 days from the oviposition.

The worker bees carry out the broadest range of tasks necessary to the colony's well-being. The life of the workers is characterized by the so called temporal polyethism, which means that the same individual passes through different forms of task specialization as it grows older (Sammataro and Avitabile, 2011). Their activities can be divided into two major stages: those accomplished inside the hive and those that take place outside the hive. Generally, the division of chores follows an age progression, *i.e.* inside bees are younger and outside bees are older. Anyway, this phenomenon is not an irreversible process, in fact depending on the needs of the colony workers can switch jobs, which enable the bee colony to thrive during difficult or variable conditions.

Upon emerging from its natal cell, a worker bee begins its life with cleaning activities. Cell cleaning involves removal of debris, faecal matter and the residues of old cocoons, preparing the cells for

receiving either new eggs, or pollen, nectar and honey. The next tasks, accomplished by workers of 5 to 15 days old are feeding the brood and the queen. During this activity the workers are aptly named nurses. In fact, at this point the worker honey bee have fully developed hypo-pharyngeal and mandibular glands, which are necessary for the production of royal jelly. Almost overlapping the nourishing stage, usually between 12^{th} and 18^{th} day, workers develop the wax gland and care about the maintenance and construction of the combs. Other inside activity includes receiving nectar from foragers, storing and ripening honey, removing the trash, packing pollen, ventilating the hive and thermoregulation, and performing guard duty at the entrance of the hive, inspecting all the individuals that enter the hive and seeing off intruders.

Worker honey bees switch to outside activity about after three weeks from hatching. The first flights serve as orientation flights in order to familiarize themselves with the surrounding landmarks of their hive as well as void faeces. Finally, when workers become foragers they fly out in search of nectar or honeydew, pollen, propolis and water. Usually foragers look smaller, because their brood food and wax gland have degenerated. It is important to remark the fine system of communication at this life stage which permits to spare haphazard search and optimize energy and time. Indeed, communication among foragers radically increases the efficiency of food-gathering activities by giving directions to each other for known water, nectar, and propolis sites. The formidable communication among foragers is based on a series of body movements called dances. Dr. Karl von Frisch won the Nobel Prize for discovering and describing the bee dance in 1973.

Factors affecting managed honey bee population

Recently, the number of managed honey bee colonies, *Apis mellifera*, has declined in both North America and Europe (Oldroyd, 2007; vanEngelsdorp et al., 2007; Neumann and Carreck, 2010). Many factors may account for it. One is the loss of forage as a consequence of agricultural intensification, which also affects other bee and wildlife species (Goulson et al., 2005; Potts et al., 2010). Another cause is the increasing relevance of pests and diseases affecting honey bees. Indeed, honey bee diseases (bacterial, fungal, viral, microsporidial), parasites (mites), predators (bears, birds, humans), and pests (beetles, moths) can adversely affect managed honey bees' productivity and survival (Morse and Flottum, 1997).

Colony Collapse Disorder (CCD) is a condition of colonies that first came to light in the United States in the fall of 2006 (vanEngelsdorp and Meixner, 2010). The condition is defined by a clear set of symptoms. These includes the complete lack of dead bees in the colony or apiary, and lack of kleptoparasites in dead hives despite the presence of surplus honey and pollen stores (Cox-Foster and vanEngelsdorp, 2009; Williams et al. 2010).

No single factor on its own can account for all losses or gains over a given period of time. Many factors can occur simultaneously and some influence one another. As part of this work is related to disease resistance toward brood diseases, the following paragraphs will describe the main factors affecting honey bee brood.

Varroa destructor

One of the major threats to health and survival of the Western honey bee is the ectoparasitic mite *Varroa destructor*, a specialized parasite of the genus *Apis* (Anderson and Truemann, 2002). Originating in South-East Asia, where it parasites *A. cerana*, in the last decades *V. destructor* made a host jump to *A. mellifera* and spread around the world (Rosenkranz et al., 2010). There are two distinct phases in the life cycle of *V. destructor* females: a phoretic phase on adult bees and a reproductive

phase within the sealed drone and worker brood cells. Female mites are transported within the colony by adult honey bees during the phoretic stage. The reproductive stage begins when female mites enter brood cells containing fifth instar bee larvae shortly before cell capping and lay several eggs. Mating takes place among siblings within the capped brood cells. From pupation to emergence of the honey bee, the mother mite and her offspring regularly feed on the host pupa internal tissues and eventually transmitting a wide range of viruses to the bee larvae, *e.g.* Deformed wing virus; Israeli acute paralysis virus; Kashmir bee virus; Acute bee paralysis virus (Di Prisco et al., 2011; Martin et al., 2012). Foundress mites must produce at least a mature male and mature female mite within the natal cell in order to produce viable offspring (Donzé et al., 1996). When the honey bee emerges from the cell, adult female mites and any fully developed offspring are released into the colony, meanwhile the male and any immature females die. Adult female mites can also disperse from one colony to another when transported on the bodies of drifting, robbing or swarming worker honey bees (Rosenkranz et al., 2010). Indeed, this efficient way of transmission and the foraging behaviour allow rapid spreading of the mite, and facilitates viral transmission to honey bees, beside inflicting nutritional stress and immune suppression (Martin, 2012; Chen et al., 2006; Chen and Siede, 2007; Rosenkranz et al., 2010; De Miranda and Genersch, 2010; Dainat et al. 2012).

In modern beekeeping, the main strategy to fight *V. destructor* infestation relies on the use of chemical and/or organic pharmaceutical products, often coupled with specific beekeeping practises to minimize the load of parasites in the colony. Such approaches, unfortunately, are not sufficient to eradicate the mite. Indeed, there is no known way to fight *V. destructor* infestation in a definitive manner. Besides, the pharmacological control of *V. destructor* can lead to some negative outcomes, *e.g*. loss of efficacy after prolonged use due to the development of mite resistance, propagation of susceptible colonies by beekeepers, increased beekeeping costs, and the occurrence of acaricide residues in bee products like honey and wax (Milani, 1999; Oldroyd, 1999; Wallner, 1999; Bogdanov, 2006). In addition, detrimental effects on honey bees have been reported as a consequence of acaricide treatments (Nozal et al., 2003; Gregorc et al., 2004; Pettis et al., 2004; Gregorc and Smodis-Skerl, 2007).

Brood diseases

Relevant bacterial brood diseases are represented by America foulbrood and European foulbrood. American foulbrood is caused by the gram-positive and spore-forming bacterium *Paenibacillus larvae*, which exists in both spore and vegetative stages (Genersch et al., 2006; Ritter and Akratanakul, 2006; Sammataro and Avitabile, 2011). The disease is transmitted by the spores, and the infected brood is killed by the vegetative stages, when the spore germinates in larval guts (Sammataro and Avitabile, 2011). This is the most destructive of the brood diseases and it is recognizable by irregular and sunken cappings, by change in brood colour which appears dark and "melted" down, and pupae's tongues stick out (Sammataro and Avitabile, 2011).

The European foulbrood is caused by the gram-positive bacterium *Melissococcus plutonius.* It is commonly found in weak or stressed colonies. The disease is usually prevalent in the spring, slowing the growth of the colony, but may disappear with the onset of a good honeyflow (Forsgren, 2010; Sammataro and Avitabile, 2011). Bacteria infect younger, uncapped larvae, turning them brown (Sammataro and Avitabile, 2011).

The primarily fungal pathogens are *Ascosphaera apis*, cause of chalkbrood disease, and *Aspergillus spp*., also known as stone brood disease (Morse and Flottum, 1997). Chalkbrood and stone brood diseases cause very similar symptoms. Those are non-homogeneous brood patterns and the appearance of white, mummified larvae, at the bottom of cells and on the bottom board of the hive (Contessi, 2015; Sammataro and Avitabile, 2011).

Also viral diseases can affect brood, as Sacbrood Virus (SBV). This virus infects larvae orally. The symptoms are the change in colour of the larvae which darken from white to yellow, eventually turning dark brown. Older larvae develop leathery skin and dark head regions. Black-headed larvae bend toward cell centre, failing to pupate and die with head stretched out (Sammataro and Avitabile,

2011; Contessi, 2015). Diseased larvae are easily removed in liquid-filled sacs. Usually, the disease is often seen in the spring and early summer months.

A part from American foulbrood, brood diseases can be controlled keeping strong colonies and kept under control applying beekeeping procedures like re-queening the colony, but it is clear that all this practises are time and resource consuming for the breeders (Sammataro and Avitabile, 2011; Contessi, 2015).

Breeding and selection in the honey bee

Active management and breeding of honey bees became possible thanks to the discovery of the "bee space" and the movable frame in the mid-1800s, as well as the invention of the honey extractor and the queen excluder (Langstroth, 1853; Langstroth, 1867; Dadant, 1975). The cornerstone in bee breeding was the development of practises for the production of large number of queens from a single mother queen (Doolittle, 1889). This allowed to propagate on a large scale queens from superior colonies by grafting worker bee's larvae (Oxley and Oldroyd, 2010).

As for other productive livestock, also in beekeeping there are traits that can be improved by means of artificial selection. Phenotypes are generally measured on the colony, which consists of one queen and her daughters, *i.e.* thousands of workers. The queen is the only fertile female of the colony and mates with 10 to 20 drones just once, and stores a life-lasting reserve of sperm cells in her spermatheca. Drones are haploid and their gametes are clones of their genotype, which represent half of the genetics of their mother queen. Furthermore, drones die right after mating, which means they mate just once in their lifetime and only with one queen. One of the basic principle in selective breeding is the control of mating. In honey bee this can be accomplished in two ways. The first is by means of artificial insemination of the queens, but this practise requires specific equipment and expert operators. The second is the exploitation of geographical isolation of areas (valleys or islands) in which bee populations can be controlled. In this case, one superior queen will represent the paternal line. The so-called paternal line is represented by a group of sister queens raised form one colony. This group of queens represents the unique genetics of their colony of origin as full sibs of the worker bees. These queens and their respective colonies are brought to the isolated area to produce drones. They represent the so-called drone-producing queens (DPQ). Since males are haploid, the drones produced by these queens represent the unique genetic pool of the superior colony these queens were bred from. Once the drone producing queens are actively producing males at the isolated area, virgin queens are brought to this mating station to accomplish the mating flight. Thanks to the geographical

isolation (valleys or islands) the bee breeder can control the genetics of the drones that will mate with the virgin queens. Worker bees born from a queen whose mating was controlled and pedigree is known can show three types of kinship. They can descend from the same drone, resulting in a super-sister relationship of an average of 0.75 of relatedness; they can descend from two drones born from the same DPQ, resulting in a full-sib relationship of an average of 0.50 of relatedness; or they can descend from two cousin drones born from two different DPQs and have an average kinship of about 0.35 (Tiesler, Bienefeld and Büchler, 2016). These kinship coefficients depend on the number of DPQs which were present at the mating station, which is usually known, and the number of drones the queen has mated with, which is usually set by the geneticist (Tarpy et al., 2004).

Currently, the only example of organized data collection and estimation of breeding values is represented by the German Beebreed programme (www.beebreed.eu). This system relies on a dense network of breeders which collect phenotypic data annually from about 6000 colonies which are spread more or less all over Germany, over 1000 colonies in Austria and smaller numbers in various other countries. The Institute for Bee Research (Hohen Neuendorf, Germany) is responsible for the processing of the phenotypic data, which are analysed applying the genetic evaluation developed by Bienefeld et al. (2007) to estimate breeding values for honey bees. The genetic values produced are available online to be consulted by the bee breeders. Moreover, almost in every region in Germany at least one controlled mating station is managed by professionals and it is available for the breeders who are interested in the genetic line present in that mating station. Generally, honey bees are selected for honey production, disease and pest tolerance, gentleness, low swarming tendency, high wintering ability and spring build up, and trait associated with production of beeswax, propolis and pollen import (Sammataro and Avitabile, 2011; Contessi, 2015).

In Italy, the main strategy for honey bee breeding is the mass reproduction of honey bees queens from superior queens, as no national organisation such BeeBreed exists nor laws (even if they do exist in some regional regulations) intended for the settling of controlled reproduction areas, except for small

realities near the border with Austria, where the “BeeBreed” influence shaped apiculture. Only recently, on voluntary basis we are witnessing a gradual change in the concept of setting up breeding programmes with organised collection of data on an acceptable scale and the setting up of controlled mating areas.

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Chapter 1

Hygienic behaviour in honey bees: a comparison of two recording methods and estimation of genetic parameters

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HB recording methods and genetic parameters

Abstract

Hygienic behaviour (HB) in honey bees reflects social immunity against diseases and parasites. Young bees showing HB detect, uncap and remove infested brood from a colony. We developed a new variant of Freeze Killed Brood (FKB*) test to optimise the duration of the HB test, the costs, and safety for the operator. In 2016, we performed a comparison between traditional FKB and FKB* on 25 unselected and unrelated colonies in the apiary of the University of Milano. To estimate repeatability and heritability, in 2017 and 2018, FKB* was used to phenotype, respectively, 56 and 95 colonies twice, in the context of a breeding programme. FKB* took less time and required a smaller amount of liquid nitrogen. The two methods showed a correlation between colony effects of 0.93, indicating that they measure the same trait. For single records, the phenotypic correlation between both methods was 0.64. Estimated heritability and repeatability for single records HB were 0.23 and 0.24, respectively, whilst heritability for the average HB value of two records was 0.37.

honey bee / hygienic behaviour / freeze-killed brood test / repeatability / heritability

1. Introduction

Hygienic behaviour (HB) is known as a behavioural response of honey bee workers to the spreading of infections in the colony, conferring resistance against diseases and parasites that affect, *inter alia*, the honey bee brood (Rothenbuhler, 1964; Wilson-Rich et al. 2009). Indeed, hygienic workers can detect the presence of an infected larva or pupa and react by uncapping the wax cover of a brood cell, if the cell was sealed, and by removing the diseased individual. Hygienic behaviour evolved as a general mechanism of resistance to the brood pathogens including *Paenibacillus larvae* (the causative agent of American foulbrood), *Ascosphaera apis* (the causative agent of chalkbrood), and the parasitic mite *Varroa destructor* (Gilliam et al., 1983; Spivak and Reuter, 1998; Harbo and Harris, 1999; Spivak and Reuter, 2001). Hygienic behaviour has a genetic basis, and it is a heritable trait (Rothenbuler 1964; Moritz, 1988; Kefuss et al., 1996). Therefore, it soon became an object for selective breeding programmes worldwide (Spivak 1996; Spivak et al., 2009; Büchler et al., 2010; Büchler et al., 2013). Although the relevance of genomics for bee improvement programmes is likely to increase, phenotyping remains essential. Therefore, the recording of this trait by reliable and cheap field assays is crucial to estimate breeding values.

Currently, HB is recorded by assessing the dead brood removal rate of a colony. There are two principal methods described in the literature: the mechanical killing of brood by using an entomological needle, known as the "pin-test", and the thermal killing by using low temperature through liquid nitrogen or a freezer (Newton and Ostasiewski, 1986; Momot and Rothenbuhler, 1971; Spivak and Reuter, 1998). The basic idea of the two methods is to sacrifice a determined area of sealed brood in the hive and to record how much the worker bees clean that area by removing dead larvae from it in a fixed time window, usually 24 h for the thermal killing method. The pin-test is used more frequently for its cheapness and simplicity, but it damages the pupae under the wax cap, with possible haemolymph leakage, which could affect the test result as it boosts the cleaning stimulus (Spivak and Downey, 1998; Panasiuk et al. 2008). The thermal killing procedures have a good

discriminatory ability but are more expensive regarding equipment, because of the need for liquid nitrogen or an extra trip to the apiary when a freezer is used (Espinosa Montaño et al., 2008; Büchler, 2010; Kefuss, 2015).

We developed a modification of the standard methodology described in Spivak and Reuter (1998) in the expectation that it would result in a more practical field assay and perform better. In essence, FKB* is the combination of FKB for liquid nitrogen usage and the freezer method for cutting out the brood disc. We expected a better defined test area, lower time requirement, and lower nitrogen use. In 2016, a field trial was carried out to compare the modified method (FKB*) with the standard methodology (FKB). For an improved method to be used in a breeding programme, it is important to have estimates of the repeatability and heritability of the trait. In 2017 and 2018, therefore, HB was recorded using the FKB* method on a testing population in the context of a breeding programme.

Here, we first describe the FKB* methodology for recording HB. Next, we present the results of a field comparison between FKB and FKB*, and present estimates of heritability and repeatability for HB recorded with FKB* in another trial.

2. Material and methods

2.1. Comparison of the two methods

2.1.1.Location and study colonies

The comparison was conducted at the apiary of the Veterinary Faculty of Milan, located in Lodi, Italy, during spring/summer of 2016. A total of 25 colonies were included in the field test. All colonies had good health status and were headed by naturally mated queens bought from different Italian breeders. Information on the pedigree of the colonies was not available; therefore, we assumed that these colonies were unrelated. Each colony was kept in a Dadant-Blatt hive box with ten frames.

2.1.2.FKB

The FKB method was described in Spivak and Reuter (1998). The materials needed for this test are a tube, liquid nitrogen, a camera (optional), and safety equipment. The method consists in extracting a capped brood comb from the colony to be tested, and finding a suitable portion of brood to maximise the number of capped cells covered by the tube. Then, the tube is twisted on the comb until the mid-rib of the frame. Ca. 300 ml of liquid nitrogen is poured into the tube to freeze-kill the brood delimited by the tube. Once the liquid nitrogen is evaporated and the tube is thawed, the tube is extracted from the comb and a photo is taken. The comb is marked to be easily distinguishable and repositioned in the colony of origin. After 24 h the same comb is taken out from the hive and the treated area of brood is photographed for the count of removed brood.

*2.1.3.FKB**

The key feature of FKB* is that the brood area to be tested is cut out and frozen through immersion in liquid nitrogen in an insulating bowl, rather than on the comb directly. Therefore, FKB* is only compatible with wax foundation brood combs.

The material needed for this method is a thin metal tube with a sharp end, liquid nitrogen, a camera (optional), safety equipment (insulating gloves and safety goggles), tweezers, water-based ink marker, and an insulating polystyrene box. The tube must have a diameter that allows it to pass between the iron wires of the frame (ca. 6-8 cm, depending on the frame type). A comb is taken from the hive to be tested, a suitable portion of capped brood (of any age) preferably on both sides of the comb is found, and the tube is twisted in the brood in order to pass through the comb and to cut out a brood disc. A good practise is to mark the brood disc with the water-based ink marker to track back its original position and orientation. The liquid nitrogen is poured into the polystyrene box. The brood disc is taken out from the tube and dipped in liquid nitrogen. After ca. 2 min, the brood disc is fished out using tweezers, allowed to thaw for 3 min, and then repositioned in the brood comb. A photo is taken of both sides to permit the count of sealed cells at time zero. The comb is marked on the top and placed back in the hive. After 24 h the same comb is taken out and a photo of each side is taken for further analysis of dead brood removal. If many colonies need to be tested, we suggest to place inside the insulated box cardboard walls in order to subset the internal part of the vessel. In this way it is possible to keep track of the brood discs that are dipped together in the liquid nitrogen. See ***Figure S1*** of supplementary materials for an illustrated step-by-step guide.

2.1.4.Experimental design

The two methods were applied at the same time to the same comb in each colony, where the location of both was random. We chose this approach to minimise the potential variation due to the comb and to the distribution of worker bees in the colony. The tests were repeated six times during the spring/summer of 2016. During the experiment, the composition of the tested colonies sometimes changed due to swarming and to hive condition (presence of capped brood). Therefore, not all 25 colonies were phenotyped for every replicate, but the number of replicates per colony ranged from 2 to 5. In total, 74 observations for each method were available for the subsequent statistical analysis. The time spent per test and colony was recorded.

2.1.5.Photo analysis and HB scoring

The counting of dead brood removal was performed by analysing the pictures that were taken for each tested area at time zero and after 24 h. Image analysis was performed with the help of the counter tool of the software ImageJ (Schneider et al. 2012). HB was recorded as the proportion of removed dead larvae in 24 h:

$$HB = 1 - \frac{sealed\ cells\ T24}{sealed\ cells\ T0}$$

HB was scored in the most conservative way, *i.e.*, if the cell was only partly uncapped or if it was uncapped but the dead larva was only partially removed, the cell was considered sealed.

2.1.6.Statistical analysis

The objective was to compare the HB results of the two methods in terms of average HB values, repeatability of both methods, and correlation between the two methods. In addition, to quantify the benefit of repeatedly recording HB, we derived the accuracy of the mean HB score of a colony, as a function of the number of records.

A paired-sample *t* test was conducted to compare the average HB value of a colony recorded with the two methods.

To estimate repeatability, a univariate approach was adopted for each method. The following mixed model was fitted to the data:

$$y_{ijkl} = \mu + B_i + T_j + C_{ki} + e_{ijkl},$$

where μ represents the overall mean, B is the fixed effect of the i^{th} breeder of origin (i = 1, 5) and T term represents the fixed effect of the j^{th} replicate (j = 1, 6); C represents the random effect the k^{th} colony within the i^{th} breeder (the size of k varies between breeders of origin from 2 to 7), and e represents the random error term of the l^{th} observation, where l varies between colonies from 2 to 5 due to swarming and capped brood availability.

Interest is in the *C* term, which represents the HB effect of the colony including all genetic and permanent environmental effects, whereas the *e* term represents the temporary environmental effect and measurement error (*e.g.*, due to the location of the tube on the comb). The effect of the breeder of origin was included as fixed term to avoid the inflation of the *C* variance component due to the differences between the genetic sources of the colonies. The colony effect and the error were assumed to follow a normal distribution with means zero and variances σ^2_C and σ^2_e, respectively.

Repeatability (r), which is the correlation between repeated records on the same colony (Falconer and Mackay 1996), was estimated for each method by the following formula:

$$r = \frac{\sigma_C^2}{\sigma_C^2 + \sigma_e^2} = \frac{\sigma_C^2}{\sigma_P^2}$$

where σ_P^2 is the phenotypic variance for a single records. The repeatability also measures the reliability of the estimated *C* value of a colony, based on a single record (Falconer and Mackay 1996).

In addition, a bivariate analysis was conducted to estimate the correlation between the *C* terms of the two HB scores, applying the model mentioned above. Note that the *C* term represents the colony effect of interest, so we measured the similarity of both traits by r_C rather than the phenotypic correlation r_P. If r_C is close to one, both methods essentially represent the same trait, apart from temporary measurement error (*e*).

Furthermore, we calculated the accuracy of each method as a function of the number of records. Our interest is in *C*, and the phenotype is the mean of *n* repeated records, $\bar{y}$. Thus, the accuracy is defined as the correlation between *C* and $\bar{y}$. The relationship between the accuracy and *n* reveals the benefit of repeatedly recording HB. The accuracy for each method was calculated by the following formula (see APPENDIX for the derivation):

$$a = \frac{\sigma_C}{\sqrt{\sigma_C^2 + \frac{\sigma_e^2}{n}}}$$

The trends of the accuracy of each method and the phenotypic correlation between the two methods were plotted as a function of the number of records.

Statistical analyses were performed using the computing environment R (R Core Team, 2015). Mixed models were fitted using the R package lme4 (Bates et al. 2015).

2.2. Estimation of heritability and repeatability

2.2.1. Colonies and phenotyping

Heritability and repeatability were estimated only for the FKB* method, from data collected in 2017 and 2018. FKB* was used to phenotype a cohort of 151 colonies made available by Melyos, an Italian bee-breeding and beekeeping company. The colonies were kept in two apiaries, near Zelo Buon Persico, Lodi, Italy, during 2017 and in one apiary in 2018 located in Lesmo, Monza, Italy. The tested group was composed of colonies headed by groups of sister queens with known pedigree, all naturally mated at an isolated mating station hosting one paternal line which was different for the two groups tested in the 2 years. Each colony was managed in the context of a breeding program, therefore in the most standardised way. The colonies were phenotyped twice for HB during productive season of 2017 and of 2018.

2.2.2. Statistical analysis

For the analysis, estimates of the genetic relationships between groups of workers and queen in colonies are required, and also those with the groups of drone-producing queens with which queens are mated. We used the methods of Brascamp and Bijma (2014) to estimate these relationships. The pedigree file was built following the procedure described in Brascamp et al. (2016). To estimate heritability and repeatability, the statistical package ASReml and the pin function of the nadiv package were used in the computing environment R (Butler, 2009; Wolak, 2012; R Core Team, 2015). Only the genetic effect of the workers was included, as the paucity of data did not allow us to

simultaneously estimate the queen and the workers effect. Following Brascamp et al. (2018), we used the additive genetic variance of worker *groups* to calculate phenotypic variance.

First, we fitted the overall average of HB for each colony, using the following mixed model:

$$y_{ijk} = \mu + ApY_{ij} + A_{w_{ijk}} + e_{ijk}$$

where μ represents the overall mean, *ApY* is the fixed effect of the combination of the i^{th} apiary (i = 1, 2, 3) and the j^{th} year (j =1, 2); A_w represents the random genetic effect of the k^{th} colony where the number of colonies per apiary varies between 17, 39, and 95, 151 in total (k = 1, 151) and *e* represents the random error term. This model allowed us to estimate the heritability of the mean value of two HB records measured with FKB*.

Secondly, we fitted a repeatability animal model:

$$y_{ijklm} = \mu + ApTY_{ijk} + A_{w_{ijkl}} + pe_l + e_{ijklm}$$

where μ represents the overall mean, *ApTY* is the fixed effect term representing the combination of the i^{th} apiary (i = 1, 2, 3) and j^{th} recording time (j = 1, 2) and the k^{th} year of observation (k =1, 2); A_w represents the random genetic effect of the l^{th} colony (151 colonies in total), *pe* represents the random permanent environmental effect of the l^{th} colony (l = 1, 151), and *e* represents the random error term. This model allowed us to estimate both heritability and repeatability for HB.

In order to compare the two models, we inspected the accuracies of estimated breeding values of colonies for both methods.

3. Results and discussion

3.1. Comparison between the two methods

The aim of the first part of this study was to compare only FKB* with the standard FKB. However, other methods are available to measure dead brood removal ability of a bee colony (Büchler et al., 2013). Table I reports some practical aspects of recording HB with FKB* compared to FKB. FKB* was found to take less time in the field, since no evaporation time is required. On the other hand, FKB* requires the analysis of four instead of two pictures for HB calculation. Concerning materials, FKB* required less liquid nitrogen, because it is possible to freeze many brood discs at the same time, repeatedly using the same liquid nitrogen since it is kept in an insulating box. FKB* is safer because it requires less handling of liquid nitrogen, reducing the chance to get burned by accidental spilling. Moreover, FKB* requires only one tube (or a few if many colonies have to be recorded simultaneously and more than one operator is performing the test).

A visual comparison of the two methods is shown in Figure 1. It can be noticed that FKB* produced clear borders of the killed area on both sides of the brood frame with no evidence of collateral brood damages (Figure 1 c, d; blue circles), giving complete control over the amount of killed brood. Figure 1 also shows that FKB, which is carried out only on one side, is capable of killing the brood on the other side of the treated area. Indeed, in Figure 1, a large patch of removed brood is visible in correspondence of the FKB which was carried out on the other side of the comb.

An empirical feature was that the bees, regardless of their HB score, tended to clean perfectly all the brood that was physically and irremediably damaged by the tube. We observed this phenomenon in both methods. As described by Spivak and Downey (1998) and Panasiuk et al. (2008), we also noticed that the mechanical injury may trigger the stimulus of dead removal. Therefore, in our HB estimation, we did not consider the cells on the circle in both methods.

Comparing the results of the two recording methods, we found that HB measured with FKB (m = 0.59, sd = 0.21) was significantly lower than with FKB* (m = 0.70, sd = 0.17) with an estimated mean difference of -0.11 (t = -4.80, df = 24, P < 0.001). This result indicates that on average HB score is higher if measured with FKB* compared to FKB. The consistent lower score produced by FKB test could be due to the fact that the standard test is killing a broader area compared to the one considered for the calculation of HB. Therefore, they may spend time cleaning outside the tested area lowering the HB result. These collateral damages are represented by the surroundings of the tube which can be killed by cold fumes whilst liquid nitrogen is evaporating and by the correspondent brood on the other side of the comb which can be killed by the deep freezing treatment.

Table II reports the estimated variance components from the univariate model, and correlations from the bivariate model. Results of the univariate model show that almost half of the total variance is explained by the effect of the colony, in both methods. Repeatability was slightly higher for FKB* (0.48) than that for FKB (0.42). Both estimates are close to the value of 45.5% reported by Bigio et al. (2013), who repeated the FKB test 10 times on a cohort of 19 unrelated and unselected colonies.

The correlation of the colony effects was very high (0.93), which implies that the two recording methods essentially measure the same trait. Indeed, the correlation of the colony effects comprises all genetic and permanent environmental effects of HB. The phenotypic correlation between single observations with the two methods was clearly lower (0.63), which indicates that the correlation of the temporary measurement errors (0.42) is much lower than the correlation of colony effects.

The phenotypic correlation in Figure 2shows the similarity between the two methods as a function of the number of records. Repeating the test increases the similarity between the two recording methods, and with many replicates, the phenotypic correlation asymptotes to a maximum equal to the correlation of the colony effects ($r_c = 0.93$). Therefore, if the test is repeated many times on a colony, the probability to assess the true merit of a colony increases, regardless of the recording method. This can also be seen from the trend of the accuracy for each method shown in Figure 2. The accuracy

represents the correlation between the mean of the phenotype measured *n* times and the true effect of the colony, *i.e.*, the permanent component of the trait for each recording method. The accuracy increases strongly between 1 and 4 observations. These values are directly linked to repeatability (APPENDIX). For each method, repeating the test at least twice is highly advisable for a more accurate estimate of the HB level of a colony, as illustrated in Figure 2

The estimates for the environmental effects for each method are represented by the residual variances in Table II. The residual variance for FKB* (0.013) is almost halved compared to FKB (0.022). Moreover, the correlation between the environmental effects between the two methods (0.42) indicates that temporary variation in the two recording methods is similar but not identical. The lower environmental variance of FKB* compared to FKB suggests that FKB* could be successful in eliminating unwanted sources of environmental variation. An example could be the collateral killing that occurs with FKB due to the lack of a clear border of the killed area on the other side of the comb (Figure 1c).

Table I Practical aspects of the FKB and FKB* methods.

	FKB	FKB*
Liquid Nitrogen	~ 3 colonies/l	~ 8 colonies/l
Time	15 - 20 min/colony	7 - 10 min/colony
Tubes (cylinders)	1 for each colony	1 for all colonies
Tested area	1 side	2 sides
Photo analysis	2/colony	4/colony

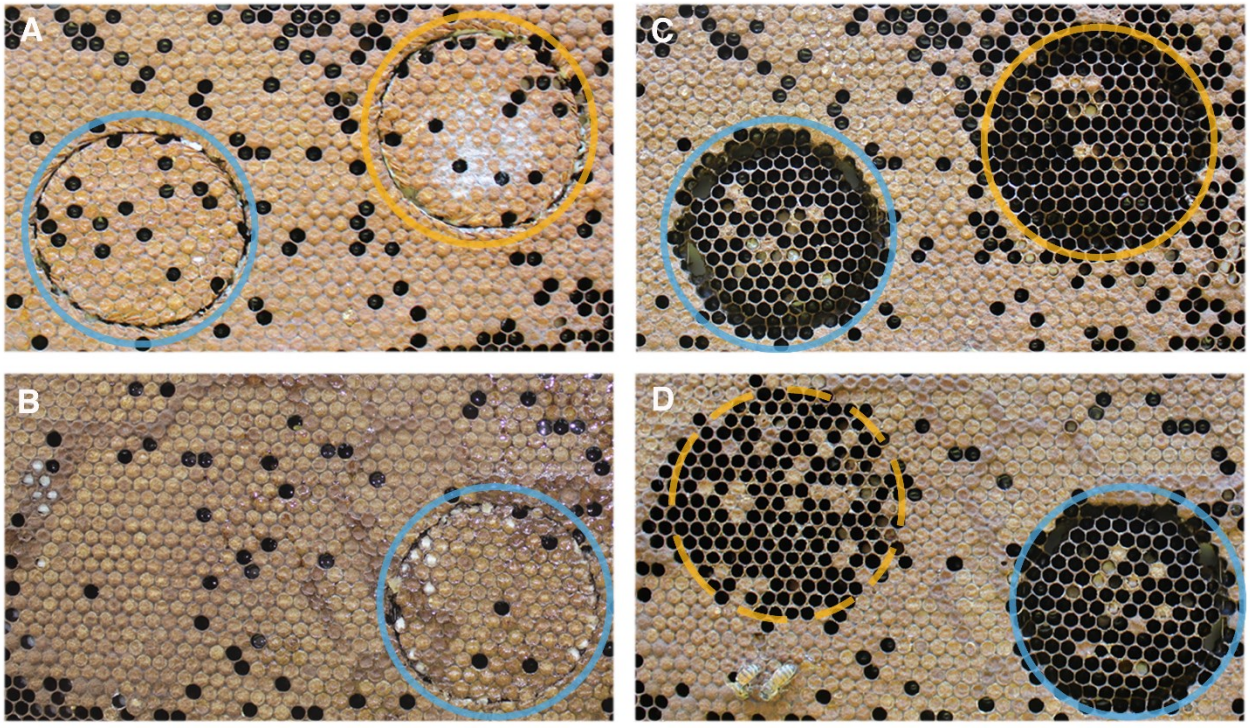

Figure 1. Visual comparison of the two methods. The pictures show the two sides (namely A and B) of a tested comb where the two methods were performed simultaneously. Blue circles surround FKB*-tested brood disc, and orange circles surround FKB-tested brood area. **a**) side A of the comb at time zero, which shows the brood disc that was cut, frozen, and repositioned for FKB* (blue circle) and only frozen on the comb for FKB (orange circle). **b**) side B of the comb at time zero, which shows the same portion of comb in picture A but on the other side. **c**) side A of the same comb after 24 h from the test. **d**) side B of the same comb after 24 h from the test. The large patch of removed brood corresponds to the treated area of FKB carried out on side A (orange dashed circle).

Table II Estimated variance components for HB recorded with the standard method (FKB) and the variant method (FKB*). Variances (Var) of random colony effect (C), residual (e), and total (P) estimated with univariate model are used to derive the repeatability (r) for each method. Phenotypic correlation (r_P), correlation of colony effects (r_C), and correlation of error term (r_e) were derived from variance and covariance components estimated with the bivariate model. Approximate standard errors are reported in brackets.

	FKB	$FKB*$
$Var\ (C)$	0.016 (0.008)	0.012 (0.005)
$Var(e)$	0.022 (0.005)	0.013 (0.003)
$Var(P)$	0.038 (0.008)	0.024 (0.005)
r	0.42 (0.15)	0.48 (0.13)
r_P	0.64 (0.09)	
r_C	0.93 (0.13)	
r_e	0.42 (0.12)	

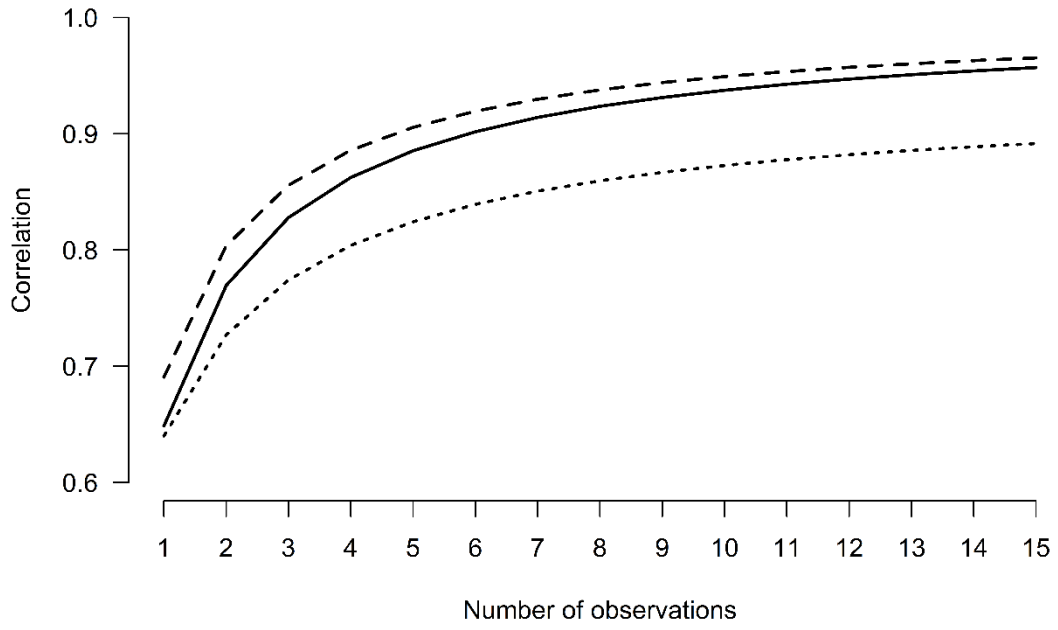

Figure 2. Correlations for mean of repeated HB records on a colony as a function of the number of observations. Dotted line: phenotypic correlation between FKB* and FKB calculated with parameters from bivariate model; solid line: accuracy for FKB calculated with parameters from the univariate model; dashed line: accuracy for FKB* calculated with parameters from the univariate model.

3.2. Heritability and repeatability

Table III shows the estimates for the variance components and the resulting heritabilities and repeatability of HB recorded with FKB*. As expected, heritability for the average HB score of two records (0.37 ± 0.25) was higher than the one estimated with the repeatability model (0.23 ± 0.16), but it is smaller compared to the value of 0.65 ± 0.61 reported by Harbo and Harris (1999) and to the values of 0.56 and 0.57 recently published by Guarna et al. (2017). The higher heritability is explained by the fact that in the average model, the dependent variable was the average of two HB measures. Therefore, the total resulting variance was smaller (0.018) than for single records (0.029).

The estimated permanent environmental variance was close to zero (2.7×10^{-4}). Therefore, the repeatability estimate was near to heritability (0.24, Table III).

To compare the two models, we computed accuracies of estimated breeding values for each model which appeared to be very similar suggesting that there is in principle no benefit of a repeatability model over an average model.

Table III Estimated genetic parameters for hygienic behaviour. Variances (*Var*) of genetic effect for the average of workers (*w*), permanent environmental effect (*pe*), residual (*e*), and phenotypic (*P*). Derived from these are estimates of heritability (h^2) and repeatability (r). $\bar{r}_{\hat{A},A}$ is the average accuracy of breeding values. Approximate standard errors are reported in brackets

	Average model	*Repeatability model*
$Var\ (w)$	0.007 (0.005)	0.007 (0.005)
$Var\ (pe)$	-	0.0003 (0.0039)
$Var\ (e)$	0.011 (0.004)	0.022 (0.003)
$Var\ (P)$	0.018 (0.002)	0.029 (0.003)
h^2	0.37 (0.25)	0.23 (0.16)
r	-	0.24 (0.09)
$\bar{r}_{\hat{A},A}$	0.50	0.50

4. Conclusion

FKB and FKB* are two ways to measure the same trait, *i.e.,* the dead removal ability of a bee colony. We did not investigate in this study the correlation of HB with the biological trait of interest as previous reports showed that HB can be used as a proxy to select colonies for resistance to main brood pathogens (Spivak and Reuter, 2001a; Panasiuk et al., 2008; Panasiuk et al., 2014). FKB* requires less time and liquid nitrogen, and has a smaller measurement error, resulting in a slightly higher repeatability. To accurately measure HB, the test should be repeated at least twice. Heritability for the average HB score of two FKB* recordings was 0.37, indicating good prospects for genetic improvement of HB. Based on accuracies of estimated breeding values (EBVs), there was no benefit of using a repeatability model over the use of a model for the average of two HB score.

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Authors' contribution

EF and RR conceived, planned and carried out the experiments. GP and RR contributed in the design and supervision of the project. EF, PB, RR and EWB contributed to the analysis and interpretation of the results. EF drafted the manuscript and designed the figures and tables. PB, RR and EWB provided critical feedback on the manuscript and participated in the revision of it. All authors read and approved the final manuscript.

APPENDIX

In this appendix, we derive the accuracy as the correlation between the colony effect (C) and the average HB value measured on a colony with n observations.

$$a = Corr(\, C, \bar{y}\,)$$

Applying the definition of correlation, it follows that:

$$a = \frac{Cov\,(\,C,\ \bar{y})}{\sqrt{Var\,(C) * Var(\bar{y})}}$$

Considering that $\bar{y} = \ \mu + C + \ e/n$, where n indicates the number of observations and assuming no interaction between the genotype and the environment, it follows that

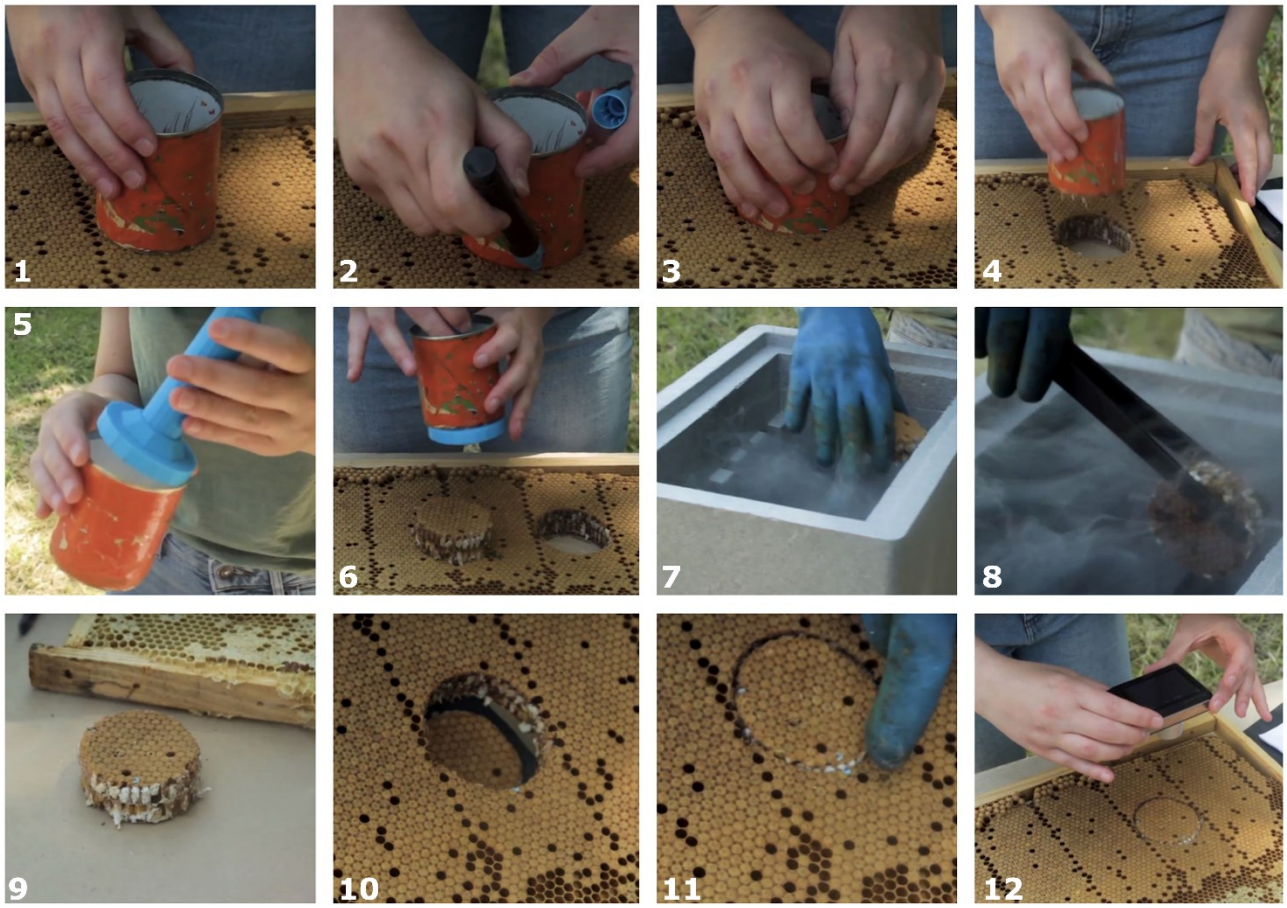
$$a_n = \frac{\sigma_C^2}{\sqrt{\sigma_C^2 \times (\sigma_C^2 + \frac{\sigma_e^2}{n})}}$$

Division of numerator and denominator by σ_C gives

$$a = \frac{\sigma_C}{\sqrt{\sigma_C^2 + \frac{\sigma_e^2}{n}}}$$

Supplemetary materials

Figure S1 Step by step illustrated procedure of FKB*. 1: find suitable capped brood area, preferably on both sides of the brood comb and choose an optimal area where capped brood are preponderant, place the metal tube over the brood; 2: keep track of the original position of the brood disc by marking the brood with a water based ink marker both inside and outside the tube; 3: twist the tube in the brood and pass through the comb to cut out the brood disc; 4: extract the tube; 5: example of a 3d printed piston, in alternative a plastic glass can be used; 6: use a piston to pull out the brood disc from the tube; 7: dip the brood in liquid nitrogen kept in the insulated box; 8: after ca. 2-3 minutes fish out the brood disc with tweezers; 9: wait ca. 2-3 minutes for the thawing of the brood disc; 10: place back the brood disc in the comb; 11: make sure to place the brood disc in the original position with the aid of the markers; 12: take pictures right after the test (time 0) and after 24 hours (time 24) to estimate the amount of dead brood removed.



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Chapter 2

DNA extraction from queen's wings as a suitable approach for genotyping – Preliminary results

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Queens' wings genotyping

Abstract

In livestock, genomics has been applied since a decade in combination with phenotypic information for the estimation of breeding values. In honey bees (*Apis mellifera*), the advantage for including genomics in selective breeding programmes is represented by potential higher genetic gain. DNA extraction methods for small animals such as insects often rely upon destructive approaches. The challenge was to develop a tissue sampling method which permitted the survival of the animal while providing adequate quantity and quality of DNA for genotyping. Along with previous reports of DNA extraction from several specimens, this study aims to contribute to the development of a suitable methodology for genotyping honey bees' queens using DNA extracted from wing cuttings. A total of 55 queens with known pedigree were enrolled for this study. Polymerase chain reaction (PCR) was performed on candidate genes for hygienic behaviour and the products were sequenced. The extracted material was sufficient for PCR amplification of candidate genes. Preliminary analysis of the sequences resulted in the discovery of about 2700 variants (SNP/Indel). Our results show that it is possible to extract DNA from wings' cuttings permitting to implement genomic approaches in honey bee breeding programmes.

1. Introduction

In the last decade, genomics has been implemented worldwide in livestock breeding programmes with successful results in many species (Pollak et al., 2012; Duchemin et al., 2012; Preisinger et al., 2012; Ibáñez-Escriche et al., 2014). Compared to traditional selection programmes based on pedigree and phenotype, genomic selection can be valuable in increasing accuracies, shorten the generation interval and it is useful for traits which are difficult or expensive to measure, such as sex-related traits or traits that can only be measured in non-optimal conditions for the animals like disease resilience or carcass quality (Hayes et al., 2013). In honey bees, the impact of the application of genomics was very recently explored by Brascamp et al. (2018). The advantage for including genomics in bee-breeding programmes is represented by the possibility to reduce the generation interval significantly and increase the accuracies of estimated breeding values resulting in higher genetic gain (Brascamp et al. 2018).

The genome of *Apis mellifera* (approximately 300 million base pairs, available on Genbank) was recently sequenced by the National Human Genome Research Institute, part of the National Institute of Health (NIH) and made public (Weinstock et al., 2006). Therefore, in the last few years we are witnessing the investigation and application of molecular markers related with traits of particular interest for beekeeping (Lapidge et al., 2002; Gramacho et al., 2003; Oxley et al., 2010; Le Conte et al., 2011; Behrens et al., 2011; Tsuruda et al., 2012; Spötter et al., 2016).

The main obstacle of applying molecular studies in bee-breeding programmes is to sample biological material from the only long-lasting reproductive individual of the colony, *i.e.* the queen. The clipping of the queen wings in professional beekeeping is a common practice and it ensures the survival and normal activities of the animal (Forster, 1971). With the availability of commercial DNA extraction kits, very sensitive to low amounts, it is now possible to implement methodologies to genotype insects from small biological specimens. Previous reports showed that it is possible to extract DNA from a

very small tissue sample, such as wing tips, in sufficient quantity for microsatellites studies (Chaline et al., 2004; Gregory and Rinderer, 2004; Gould et al., 2011).

Hygienic behaviour is a trait of interest since it confers brood disease resistance at the colony level (Wilson-Rich et al., 2009). Despite the hygienic phenotype is deeply investigated by the scientific community, hygienic behaviour is still poorly characterized from a molecular perspective. In order to have a biological sample for molecular analysis and at the same time maintaining the animal alive, we verified if sampling wings clipping and extracting DNA from this specmen could give enough material to be used for the discovery of molecular markers that could be useful in bee-breeding by next generation sequencing (NGS) techniques. In recent years, there has been substantial progress in the selection of hygienic behaviour in honey bees (Büchler et al., 2010; Rinderer et al., 2010). Selective breeding yielded at least two documented honey bee stocks, Minnesota Hygienic Bees and Russian Honey bees, which are currently used in beekeeping in the United States (Rinderer et al., 2001a; Rinderer et al., 2001b; Ibrahim and Spivak, 2006). Recent studies have determined hygienic behaviour to be a quantitative trait and up to seven loci may be responsible for its expression (Lapidge et al., 2002; Gramacho and Spivak, 2003; Oxley et al., 2010; Danka et al., 2011). A genome-wide association study (GWAS) was also presented by Spötter et al. (2016) to find genetic markers associated with defensive behaviour against *Varroa destructor*. Genomic approaches require the genotyping of a large set of individuals to detect reliable effects of low or moderate magnitude. Unfortunately, this is not always possible in the case of in field phenotype collection of honey bees. The targeted resequencing of candidate genes is an alternative approach which shows higher statistical power and chance of success when dealing with limited sample size, since multiple testing is reduced, as the number of independent statistical tests is lower than what is assumed for genome-wide statistical analysis (Amos et al., 2011). Therefore, we decided to use the target sequencing of candidate genes as a tool for discovering molecular markers potentially associated with hygienic behaviour with the perspective of implementing molecular tools to fasten the selective process and increase accuracies in the breeding values estimation in honey bees.

2. Materials and methods

2.1. Animals and biological samples

The queens enrolled for this study were part of the breeding population analysed for hygienic behaviour in 2017 (described in Chapter 1). Wing clippings were collected as follows: the queen was kept firmly by at least two legs on the same side while the wings were cut with a small scissor (Figure 1). For this procedure at least two operators were needed, one for holding the animal and perform the cut of the wings, the second for holding a funnel over the collection tube (a 1.5 ml Eppendorf). During the collection in the field, the wings were kept in an insulating box with coolers. Once back to the laboratory, the wings were kept at -20°C until further processing.

Sample collection was accomplished in early spring on queens who survived the winter and were starting the performance testing for the selective breeding programme.

Figure 1 Honey bee queen of *Apis mellifera*. The picture shows how wings cuttings were sampled.

2.2. DNA extraction

DNA was extracted from wings modifying the supplementary protocol for purification of total DNA from insects of the DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA, USA). The modification consists in an initial incubation of the samples with proteinase K for 20 minutes, further steps were carried out following the manufacturer's instructions (https://www.qiagen.com/fr/resources/resourcedetail?id=cabd47a4-cb5a-4327-b10d-d90b8542421e&lang=en).

For each extracted sample, the quantity (ng/µl) and quality (260/280 nm Absorbance ratio) of extracted DNA was assessed using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, St. Waltham, MA, USA). The ratio of 260/280 nm Absorbance is used as a proxy for the presence of contaminants (mainly proteins) that can be present in the final extraction liquid. An ideal ratio for DNA should range between 1.8 and 2.2. In the present work it was particularly important to check this parameter since wings have a low proportion of cells with respect to the whole biological sample and contaminants could inhibits the downstream analyses.

2.3. Candidate genes selection and fragments amplification

Candidate genes were selected by extensive literature research of potential targets known for their possible effect on HB and disease resistance (Gramacho et al., 2003; Le Conte et al., 2011; Tsuruda et al., 2012; Guarna et al., 2015; Spötteret al., 2016; Guarna et al., 2017). We selected 9 genes which resulted the most interesting from literature data and referred to a phenotyping method comparable with the method used in this work. Primers were specifically designed to amplify spanning regions of each gene (Table I). Amplicons size ranged between 800-1800 bp. For each gene, primers sets were designed using the online tools Primer 3 and PrimerDesign M (Koressaar & Remm, 2007; Untergasser et al., 2012; Brodin et al., 2013; Yoon & Leitner, 2014). All PCR reactions were carried out using GoTaq® Long PCR Master Mix (Promega Corporation Madison, WI, USA) following manufacturer's instruction. The thermal protocol for the PCR reactions was 94 °C for 2min, 34 cycles of 94 °C for 30 sec, 55°C for 30 sec, 68°C for 2 min, final elongation step 68°C for 5 min. A subset of fourteen individuals was chosen as representatives of the diversity within the population under study and PCR amplified for all the selected genes. PCR products were checked on a 1% agarose gel and subsequently sequenced as described below.

Table I Table of primers designed for the amplification of spanning fragments of each selected gene.

Name*	Forward Primer (5'->3')	Reverse Primer (5'->3')	Size (bp)
OBP18-1	TGCACAATCTGATACTGATGAG	CATCAATGTTGCCGTCACGA	1300
OBP18-2	TACGTGCTGTGGTGCCAATT	TTTCGGAAAATAAAGAGGAACAT	1173
KCAT-1	CGATCCAAAGATGTAAAATTATG	TTGCGAATAAATCTACCTGTGC	1366
KCAT-2	TGATGGAGGTTTTTTAACACG	TGGACCAATTCCCATAATACTAGG	959
KCAT-3	GAACATCCACGGCCTCAAAC	TGCTGGCATGGAAATGAACT	1338
CBP-1	GAATGAACCAATCACGATGC	ACTGTGGTATTTGTTTGATCCCA	1345
CBP-2	CAACAAGCAAGTCGTCAGAGA	CGAAATGTATAAAGCGATATATGTC	1405
GRCW-1	TGGTTACAGGAAGAATCGCG	AAAATACCTCCATGTGCGCC	1530
GRCW-2	GCGGTGGTACTAAAGGAGGT	CGAAACCACTTCCGAAACCA	1581
GRCW-3	AAGTGGAGGTGGTAGCTGTC	CTCTTGGCCACATAAGCTGG	882
OBP16-1	TTTTAAGCACCTACCTATTGACT	CGTCCTCTATATTGATTTTGCCA	1510
OBP16-2	AGATTTTCAAAATGCGATACGT	ACAGTCACCATTGCACCAAC	1207
OBP16-3	CGATATTTGTTCAGTTCTCGTTG	TTGACTAGTGCCAGTTTCGC	1206
OBP16-4	ATGTTCTTTTTAGACACATGAGG	ACAGACTCGAATGCACTTGA	1110
SPARC-1	GCACTGGGCGTTACGTAATT	AATTGTGACAAACGCCCACT	1393
SPARC-2	AGAGAGGGAAGGAAGTTGCT	TACTCGCGCGTCACAAAATT	1220
SPARC-3	TTGCTCCGTTCTTCTTGCAG	TACCTGTTGTTGAGCCGAGA	1561
SPARC-4	GCCGCAGAGATCGCAATG	GCTTCCCGTTGCATTTTCAG	1600
SPARC-5	TGTTGTTTTACTGGAGGAACGA	TAGGGAGAGGAAACGAGCAG	1582
SPARC-6	TGTCGAACCGTCATACCGAA	GGCCACTTACCGTTTATTTTCA	1587
VAMP-1	TCGTGTGTTTTACCGGCATT	CGATGTGACTGTCCTTTCTGT	1362
VAMP-2	ACCTAACCCCATTCGTCGTA	AAGGGCACTCTCTTCTTCCC	1589
VAMP-3	ACAATGACAGCTGACACAACC	ACCATGTACCACTGCAAATTACT	1520
TBP-1	CAGAATTAAGAAGAGAAGTCATCGA	GCACAAAGGAAAGGCAAATGT	1600
TBP-2	GCTGCTATTACTTTAATATCAGCGG	GAATCTTAAAATAGCAGAATGAACC	1521
CYTO-1	ATTTAAATTGATACTTACAATGTAG	CTATTAATGGAGGATTGTATCC	1689
CYTO-2	ATTTCCTCCGTAAAAAATTATATAC	TCTATCAATGTGTTGACAAAATCG	1520
CYTO-3	AATGAATTTCTCACGAATCTGATC	TTACTTAAGAAGATAATTGTTGC	1658
CYTO-4	CCAATAATGTTACTTCTTAACATC	TTCTGATAGTGTGATCCAAAATG	1826
CYTO-5	TATCGTCAAAAATAGAAATTTG	TTTTTCCAAAAAGTATATTTTGATG	1844
CYTO-6	TGGATTACTTCCAAATTCTCTGCG	TTCTTGATTTTGAGCTAACTCG	1497
CYTO-7	TTGAAACCTCTTCATCGACG	ACTTTCGTGTATTTGTCCCCTCTAG	1668

**OPB18*= Odorant binding protein 18; *KCAT*= 3-ketoacyl-CoA thiolase; *CBP*= Calcyclin Binding protein; *GRCW*= Glycine-rich cell wall structural protein-like protein; *OBP16*= Odorant binding protein 16; *SPARC*= Secreted protein acidic and rich in cysteine Ca binding region; *VAMP*= vescicle associated membrane protein; *TBP*= 26S protease regulatory subunit 6A - Proteasome subunit: similar to Tat-binding protein-1; *CYT*= probable cytochrome P450 6a14.

2.4.Library preparation and sequencing

PCR products were pooled in order to have the amplified region of interest in one tube for each sample (each animal). The final concentration of DNA for each pool was assessed with capillary electrophoresis with the Agilent DNA 7500 of the 2100 expert Bionalyzer (Agilent, Santa Clara, CA, USA). This information is essential to prepare proper dilutions for the DNA library preparation step. Libraries were prepared using Nextera XT DNA Library Prep Kit (Illumina), that supports ultra-low DNA input of only 1 ng. It enables a wide range of input samples, including small genomes, PCR amplicons greater than 300 bp, plasmids, microbial genomes, concatenated amplicons, and double-stranded cDNA.

Libraries products were prepared following manufacturer's instructions and checked with the High Sensitivity DNA Assay of the 2100 expert Bionalyzer (Agilent, Santa Clara, CA, USA). Single animal libraries were pooled and sequenced on Illumina MiSeq platform (Illumina, San Diego, CA, USA).

2.5.Sequencing data analysis

The sequencing data generated by MiSeq System were uploaded to the Galaxy web platform and the public server at usegalaxy.org was used to analyse the data (Afgan et al. 2016). Galaxy is an open, web-based platform for accessible, reproducible, and transparent computational biomedical research. This platform groups a number of useful tools to manage data produced by sequencers in a user friendly way.

The first step for analysing sequencing data was checking the quality of the obtained reads. Reads were processed to respond to minimum quality requirements for further analysis. Reads' quality indicates how trustworthy is the information generated by the instrument. Each base for each reads in the format fastq contains its quality information. The quality information is represented by the so-called "Phred" score Q. FastQC and MultiQC are tools which summarize on the overall quality of

sequencing data (Andrews, 2010; Ewels et al., 2016). Sequences with a Q value equal or higher than 25 are generally considered suitable for further processing.

Sequences were downloaded from the sequencer already trimmed from libraries and samples indexes and since all sequence bases had a Q value higher than 28 the reads were directly mapped to the reference genome of honey bee available on GenBank (version Amel 4.5) using BWA-MEM alignment algorithm (Li and Durbin, 2009; Li and Durbin, 2010; Li, 2013). Aligned reads were stored in Binary Alignment Maps (BAM) files. These type of files store the information of each aligned reads along with quality scores for the alignment. We sorted BAM files retaining the aligned reads with minimum mapping quality of 20. Variant calling on the mapped reads was accomplished with Freebayes (Garrison, 2012; Tange, 2018).

3. Results and discussion

DNA was extracted from wings clipping successfully from all samples with an average of 5.41 ± 1.22 ng/µl and 1.83 ± 0.77 260/280 absorbance ratio. All the tested samples were successfully PCR amplified, as demonstrated by the agarose gel check. An example of the results for four genes fragment is given in Figure 2.

After pooling the single PCR fragments of each individual in a single sample to be sequenced, libraries were prepared following Illumina's protocol. We obtained successfully results for all samples as shown by High Sensitivity DNA Assay of the 2100 expert Bionalyzer electropherograms. Some examples of the library products obtained are given in Figure 3. The average library size was about 500 bp.

Sequencing resulted in a total of 7,157,842 reads, where the average number of reads per animal was 511,274. Quality of the obtained reads was over 28 Phred score and a visual summary is reported in Figure 4. On average, 90.4% of the reads were mapped per animal against the reference genome (min. 77.0%, max. 96.6%).

Results from Freebayes showed 2700 potential variants with respect to the reference genome. After filtering for SNP quality, only 356 variants were retained, of which 66 Indel and 290 SNP. The number of SNP discovered in each gene was proportional to the gene length: the longer the gene, the higher the number of variants was detected (Table II). The only exception was the *GRCW* gene, which consisted of 2713 bp and showed a total of 57 variants, compared to genes with similar lengths that showed only 3 or 4 SNP (*KCAT* and *TBP1* genes respectively).

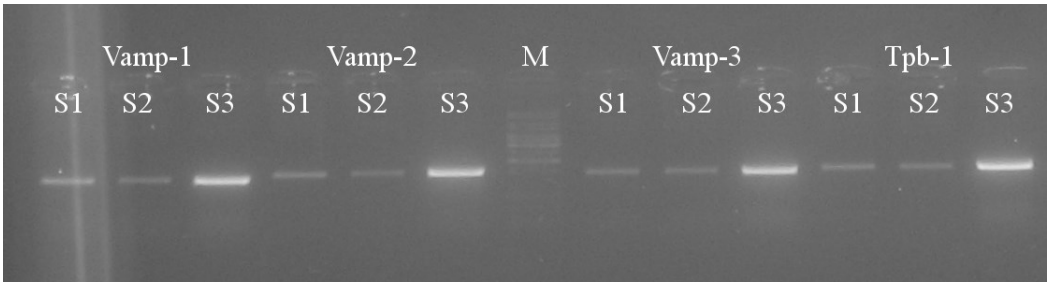

Figure 2 PCR results of the amplification of vescicle associated membrane protein (Vamp-1, Vamp-2, and Vamp-3) and 26S protease regulatory subunit 6A - proteasome subunit: similar to Tat-binding protein-1 (Tbp-1) fragments. S = sample.

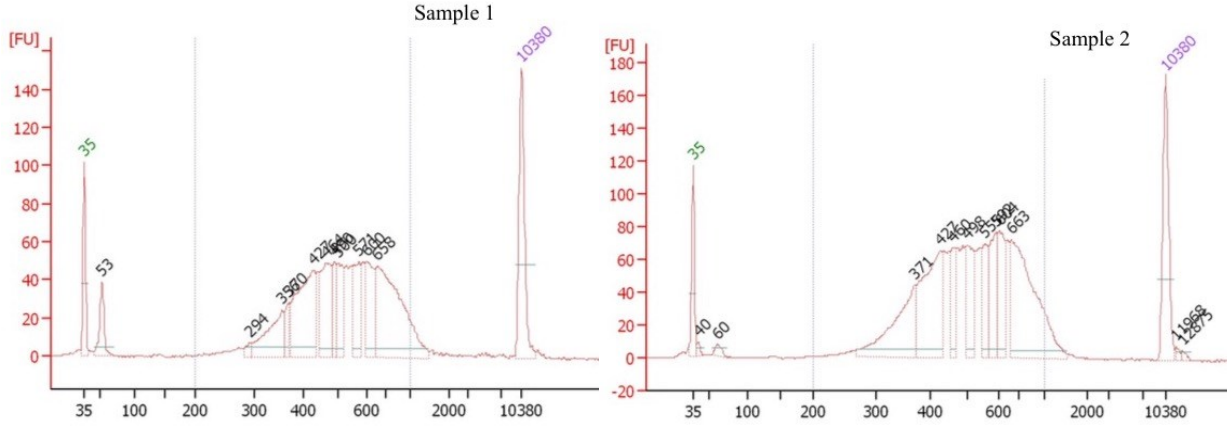

Figure 3 Two electropherogram examples of library preparation results visualized by the High Sensitivity DNA Assay of the 2100 expert Bionalyzer (Agilent, Santa Clara, CA, USA)

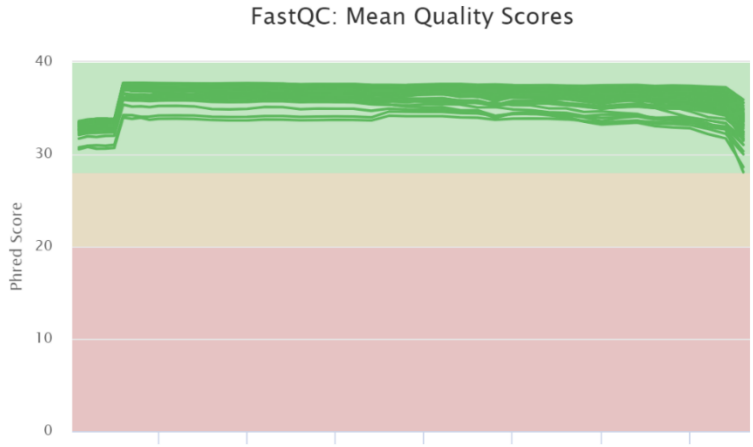

Figure 4 Sequence quality plot. Mean Phred score is reported per sample across each base position in the read.

Table II Summary of the variants discovered per gene. LG = Linkage group.

GENE	LG	Expected lenght (bp)	VARIANT	NUMBER
CBP	3	1661	SNP	1
			InDel	0
SPARC	7	6337	SNP	37
			InDel	17
VAMP	7	3316	SNP	2
			InDel	1
KCAT	10	2609	SNP	3
			InDel	0
GRCW	11	2713	SNP	51
			InDel	6
CYTO	13	10234	SNP	143
			InDel	31
TBP1	14	1952	SNP	4
			InDel	0
OBP16	15	4228	SNP	36
			InDel	7
OBP18	15	2170	SNP	13
			InDel	4

4. Conclusion

In this work we demonstrated that it is possible to obtain DNA in sufficient quantity and quality for investigating the genome of honey bee with NGS technologies. Indeed, wings' clippings, a non-lethal specimen for the insect, resulted to be a suitable DNA source even if the percentage of DNA containing tissue was very low with respect of the total biological sample mainly characterized by chitinous structures. We developed a methodology that permitted to obtain new and interesting molecular information on the genetics of the bees under study, which showed a discrete variability with respect to the reference genome even if we focused only on few genes. The next steps will be to check the gene effect of the discovered variants and their distribution in the population under study, and to analyse the association between genetic variability and HB, to verify if at least some of this markers could be useful for bee breeding.

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Chapter 3

Investigating genetic and phenotypic variability of honey bee queens: morphological and reproductive traits

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Queen Quality

Abstract

The quality of the queen may have an important effect on a colony's development, productivity and survival and queen failure or loss is considered a cause for colonies' decline worldwide. The queen quality, resulting from her genetic background, developmental condition, mating success and environment, can be assessed by some morphological measures. The aim of this study was to obtain preliminary genetic parameters of some traits that describe the quality of bee queens. An Italian breeding and beekeeping company provided 147 related queens with known pedigree bred during spring/summer of 2017 and 2018 using a standardized production system. The queen quality traits considered were body weight, weight and width of the tagmata (head, thorax and abdomen), wing length, diameter and volume of spermatheca, number of ovarioles and number of sperms in the spermatheca. An animal model was applied in univariate and bivariate analysis to obtain (co)variance estimates. Heritability of body and tagmata weight ranged from 0.46 (± 0.34) to 0.54 (± 0.34), whereas lower estimates were found for the tagmata width and wing length (from 0.13 ± 0.26 to 0.42 ± 0.32). Heritabilities estimated for the spermatheca diameter and volume, number of ovarioles and number of sperms were 0.17 ± 0.34, 0.88 ± 0.39, 0.70 ± 0.35 and 0.57 ± 0.35, respectively. Many phenotypic correlations related to size (weight and width) were high and positive, while we found weak correlations between morphology and reproductive traits. Genetic correlations showed large standard errors and are to be considered with caution.

1. Introduction

Honey bees are highly evolved social insects who live in colonies characterized by a cooperative system of brood care, overlapping generations and reproductive division of tasks (Wilson, 1971). In such fine organized bio-social structure, the queen is the only fertile female whose main duty is to lay eggs (Winston, 1987). Moreover, the queen maintains the colony cohesion through continuously producing a bouquet of pheromones which are actively spread within the nest and prevents the workers from substituting the queen and developing their ovaries. The queen is the core and most important individual of a colony (Fyg, 1961; Gauthier, 2011; Porporato & Laurino, 2013). The colony development, productivity and survival depend substantially on the health and fitness of its queen and the drones which she mated with (Nelson et al., 1983; Tarpy, 2003; Tarpy et al., 2013; Rangel et al., 2013). A bee colony is adversely affected if the queen shows any defects, or becomes ill and ceases to lay eggs (Kaftanoglu et al., 1988; Laidlaw, 1992; Bieńkowska et al., 2011; Büchler et al., 2013). Beside pollution, parasites and pathogens, failure or loss of the queen have been considered the most important factors leading to colony losses worldwide, especially outside the natural queen rearing season (Genersch et al., 2010; Brodschneider et al., 2010; Spleen et al., 2013; Vanengelsdorp et al., 2013). Poor quality queen is a factor that consistently ranks among the top reasons for bee colony failure (Vanengelsdorp et al., 2008; Vanengelsdorp et al., 2013).

There are many measures that can be correlated to a queen “quality”, which results from her genetic background, her developmental conditions, mating success and adult environment including beekeeper management (Hoopingarner & Farrar, 1959; Dodologlu & Gene, 2003; Oldroyd et al., 1990). The most intuitive are physical measures of the queen, such as the body weight, which was found to be significantly correlated with the fitness of the queen herself or colony productivity (Kahya et al., 2008; Delaney et al., 2011; Hatjina et al., 2014; Tarpy et al., 2012; Woyke, 1971; Collins & Pettis, 2013; Szabo et al., 1987; Szabo, 1973). Weight was also found to be positively associated with higher acceptance of queens in new colonies (Szabo, 1973; Yadava & Smith, 1971; Moretto et al.,

2004; Szabo & Townsend, 1974). Body weight was also positively correlated with reproductive organs of the queen such as ovaries and number of ovarioles, diameter of the spermatheca and number of stored spermatozoa (Kahya et al., 2008; Delaney et al., 2011; Woyke, 1971; Collins & Pettis, 2013; Tarpy et al., 2011).

Concerning external morphological properties, Amiri et al., 2017 concluded in his review that the body weight of a queen could represent an integrative measure of size and physiological condition. Therefore, it could be considered the most informative indicator of a queen quality. However, other morphological measurements of queen's body were investigated in the past such as the width of the first two tagmata of the adult (head and thorax) and wing length to study any possible association with reproductive organs (Delaney et al., 2011; Tarpy et al., 2012; Jackson et al., 2011; Tarpy et al., 2011). Results of these study are often ambiguous. For example, In Delaney et al. (2011), thorax width was found positively correlated with number of stored sperm and mating frequency. Meanwhile, other studies reported no correlation between thorax width and ovarioles number, ovary weight or mating number (Tarpy et al., 2012; Jackson et al., 2011; Tarpy et al., 2011).

In mated and egg-laying queen, the ovaries are the organs involved in the production of eggs. There are two of them occupying a vast part of the abdominal cavity (Winston, 1987). They consist of a bundle of ovarioles (ca. 150 each), which are long tubules which contain egg cells, nurse cells, and follicle cells (Carreck et al. 2013). Ovary development takes place soon after mating and is associated with distinct gene-expression patterns in the brain and ovaries, physiological, and behavioural changes in the queen (Richard et al., 2007; Kocher et al., 2008; Niño et al., 2013). The weight of ovaries in a mature egg-laying queen not only depends on the number of ovarioles but also on the number and developmental stage of eggs they contain (Amiri et al., 2017). The weight of ovaries was reported as one of the internal physical criteria to assess the reproductive potential of honey bee queens (Delaney et al., 2011; Gregorc & Smodiš Škerl, 2015; Gilley et al., 2003). Ovary dimensions and fertility are usually positively correlated (Tarpy et al., 2000). The number of ovarioles can be

evaluated at any time during the life of a queen (Carreck et al., 2013). Queen size, ovary size and symmetry are affected by larval nutrition, and during artificial queen production the age of larvae that are transferred into queen cells is critical (Woyke, 1971, Dedej et al., 1998) and influences also natural queen supersedure (Hatch et al., 1999; Tarpy et al., 2000). Beside the ovaries, the queen's reproductive system is equipped with the spermatheca. The spermatheca is a small spherical shaped organ which duty is to preserve living sperms after mating for a lifelong period of time (Winston, 1987). The spermatheca size is another measure of internal physical queen quality, because larger spermatheca could hold larger volume of semen (Amiri et al., 2017). Spermatheca size can be measured with or without the tracheal nets, and its diameter should be larger than 1.2 mm for high-quality queens (Hatjina et al., 2014; Carreck et al., 2013). This measurement was used as a direct estimation of the volume and as an indirect estimation of the theoretical maximum number of spermatozoa stored in spermatheca (Tarpy et al., 2012; Collins & Pettis, 2013; Tarpy et al., 2011; Carreck et al., 2013). The size of the spermatheca is influenced by rearing conditions and genetics, and it is inversely proportional to the larval age at which the queen was reared from (Tarpy et al., 2012; Tarpy et al., 2000). Queens raised from newly hatched larvae have a larger spermatheca (Hatch et al., 1999; Tarpy et al., 2000; Gilley et al., 2003). However, the spermatheca is rarely filled completely, as the occupied volume in experimental queens was reported to be on average 47% (Tarpy et al., 2012; Tarpy et al., 2011).

From a hypothetical perspective, a "high quality" queen should be morphologically defects-free, should have a large body and contain big sized spermatheca and ovaries in order to store a high number of spermatozoa and lay a copious number of eggs, preferably over 2000 eggs per day (Büchler et 2013, Porporato et al., 2015).

Although it is not difficult and seen as normal routine for the beekeepers to replace an unfit queen before the colony is weakened, introducing queen's traits in the selection program could improve colonies survivability and represents an added value to a queen bee breeder company.

We chose to measure both external and internal physical queen traits such as: total weight, separated weight and width of the tagmata (head, thorax and abdomen), length of the right forewing, size of spermatheca, number of sperms in the spermatheca, and number of ovarioles. We decided to count the number of ovarioles instead of weighting the ovaries because the weight may be influenced by the developmental stages of the eggs they contain, as pointed out by Amiri et al. (2017).

The aim of this research was to investigate phenotypic and genetic variability of the above mentioned measures for queen quality in a small population bred by a professional queen breeder in Northern Italy.

2. Materials and methods

The queens were provided by an Italian queen-breeding and beekeeping company that produces and sells about 400-600 queens per week, from Spring to late Summer. The breeding of the queens was characterized by a standardized production system and by a traceability of the pedigree. Also, this process was carried out following strict time-table by the same people during the season minimizing any potential error variance due to management practices.

This study was conducted on 147 queens bred during spring/summer seasons of 2017 (n = 70) and 2018 (n = 77). The queens were bred in groups of full sisters from 10 maternal lines in 2017, and 7 maternal lines in 2018. Furthermore, the maternal lines shared common ancestors. The analysed queens were bred in different times of the production season, reported as the ordinal number of the week of the year in which the mated queen was harvested from mating nucleus. All queens open mated in the same area within the year.

2.1. Animal sampling and transport

The newly mated queens were harvested after mating flight by the beekeeper at the mating station. They were shipped to the laboratory in suitable queen cages with candy and an apt number of attending worker bees (Figure 1, left).

During transport they were kept in a cardboard box equipped with a spacer support for the cages and holes for aeration (Figure 1, right). This transport system is the most common way to transport living bees in Italy, and it is designed to minimize the stress to the animals.

Figure 1 Left: queen transport cage; Right: box for queen transport

2.2. Freeze immobilization

For an easier handling during dissections, the queens were stored in the freezer at -20 °C for 15-17 min before being analysed. The cold environment (-20 °C) anesthetized the animals and induced them to an immobile state.

2.3. Morphological measures

After cold immobilization, the first analysis was to evaluate the exterior state of the insect for the detection of any macroscopic defects *e.g.* missing legs, wings, antennae, or any visible injury of the body. Afterwards, queens were euthanized by decapitation and processed. The way morphological characteristics were measured differed between 2017 and 2018.

In 2017, after the sacrifice, the insect was pinned onto a dissection petri dish. The petri dish was previously filled with paraffin in order to form a basal layer to permit the fixing of the animal on the paraffin surface with entomological needles. The right forewing was detached from the body and put beside the insect. The petri dish with the insect was numbered and photographed for posterior morphological measures with Image J software (Schneider et al. 2012). Every image contained a reference scale (graph paper) useful to determine the pixel/mm ratio. After the acquisition of the image, every queen was weighted on an analytical scale recording the weight of entire insect, and separately also the weight of the head, abdomen and thorax. Other metrics were recorded with

posterior image analysis as following: the width of the head was assessed measuring the distance from the two compound eyes; the width of the thorax was recorded measuring the distance between the two tegulae, the scales on the mesothorax that overlaps the root of the forewing; the width of the abdomen was assessed measuring the width of the first abdominal tergite. In 2018, the above described measurements were taken manually using a digital caliper. Figure 2 shows how measures were taken both with image analysis and manually.

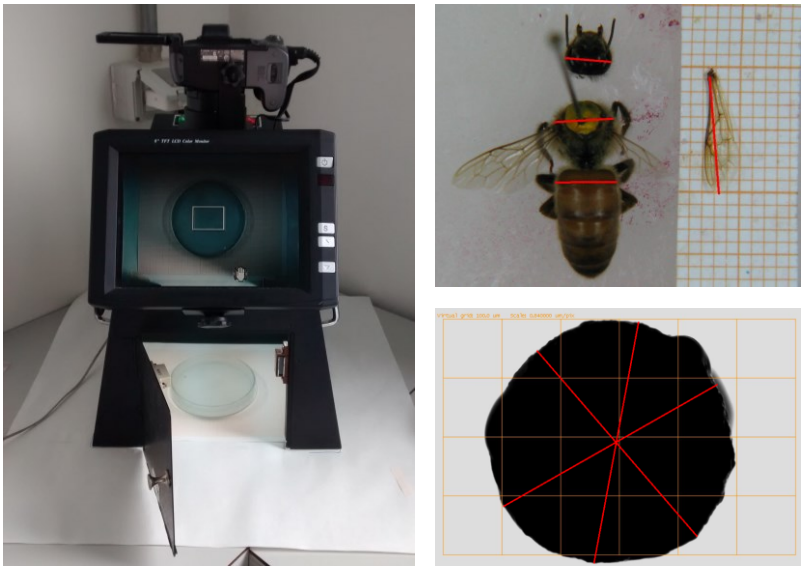

Figure 2 Left: image acquisition instrument; top right: queen tagmata width for morphological measures; bottom right: spermatheca diameter measure.

2.4. Abdomen dissection

In order to assess the reproductive characteristics of queen the abdomen was dissected following the methods described in Porporato et al. (2015). The abdomen was kept in ventral position by two proximal needles and one distal needle. Abdomen was dissected cutting the junction between dorsal and ventral tergites with a scalpel and pulling away one by one the dorsal tergites with tweezers and fixing them beside the body with needles (Figure 3). As soon as the abdominal cavity was opened, the abdomen was submerged in physiological solution (NaCl 0.9 %) to prevent the drying out of the internal organs.

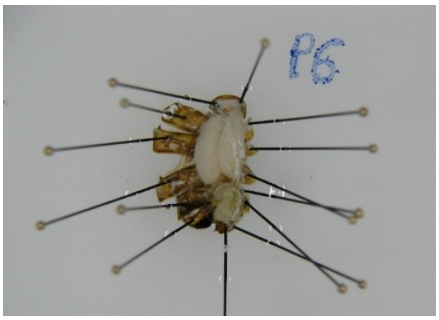

Figure 3 Queen abdominal cavity after dissection.

2.5. Spermatheca extraction and analysis

As soon as the abdomen was opened, the spermatheca was extracted and put on a glass slide. In 2017, the spermatheca was photographed on a slide under the microscope, and its diameter was measured with Image J software. In 2018, the diameters were measured by hand with a digital caliper. Since the spermatheca of a queen does not have a perfect spherical shape, the diameter was calculated as the average of three measurements. The volume of the spermatheca was calculated as the volume of a sphere.

To assess the concentration of semen, the spermatheca was popped in 1ml of physiological solution (NaCl 0.9%). The concentration of sperm was assessed using a standard haemocytometer chamber (Burker Camera) under a light microscope (Human et al., 2013).

2.6. Ovarioles count

Only the ovarioles of the right ovary were counted (Carreck et al., 2013). The number of ovarioles was estimated with a method derived from Porporato et al. (2015). The right ovary was removed from the abdominal cavity and kept in staining solution for ca. 10 minutes (methylene Blue Trihydrate, 0.5%). After dying, the ovary was washed with physiological solution (NaCl 0.9%), placed on a slide, and analysed under a stereomicroscope. The ovarioles were separated with the help of dissecting needle and counted one by one, without cutting the ovary (Figure 4).

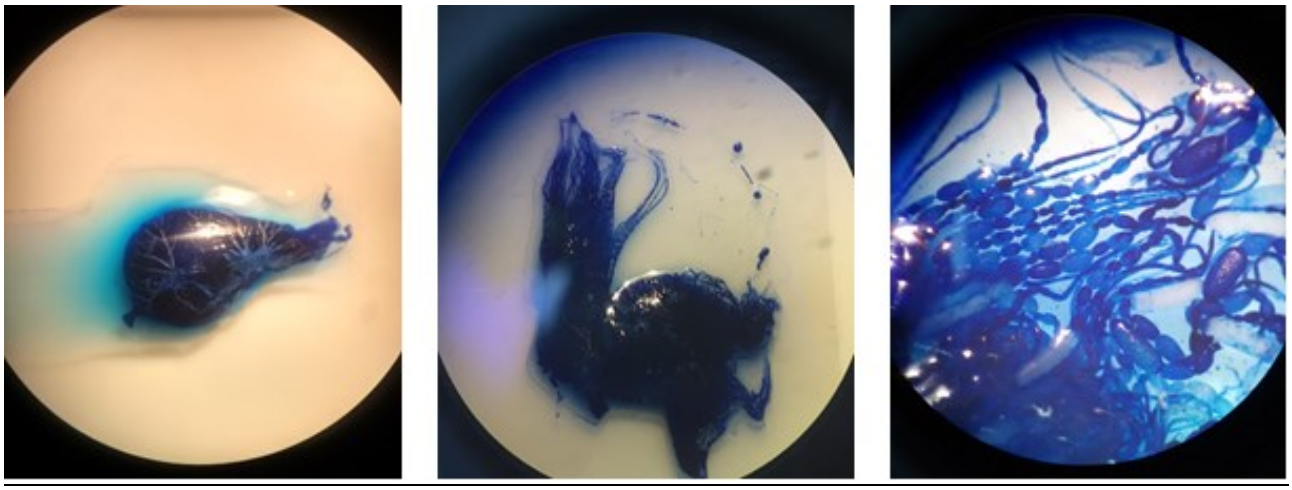

Figure 4 Ovarioles count. from left to right: an ovary after staining, an ovary during ovarioles count, detail of ovarioles' structure.

2.7. Statistical analysis

For the analysis, we used the method of Brascamp and Bijma (2014) to estimate the additive genetic relationships among the studied queens, which were full sister groups with common ancestors. To estimate heritability and genetic correlations, the statistical package ASReml and the pin function of the nadiv package were used in the computing environment R (Butler, 2009; Wolak, 2012; R Core Team, 2015).

First we fitted a univariate animal model for each trait, using the following model:

$$y = \mu + wy_i + \alpha_j + \varepsilon_j$$

Where μ is the overall mean of the trait, wy_i represents the fixed effect of the i^{th} combination of the week of the year in which the queen was harvested (i = 1, 15, specifically 6 weeks in 2017, 9 weeks in 2018), α_j represents the random genetic effect of the j^{th} queen ($j = 1,147$), $\varepsilon_{i,j}$ represents the the random error term. This model allowed us to estimate the heritability of each measured phenotype.

Secondly, we adopted a bivariate approach for each combination of trait fitting the same model above described. The bivariate approach allowed us to estimate both phenotypic and genetic correlations between the measured traits.

3. Results and discussion

3.1. Descriptive statistics

Every analysed queen was free from macroscopic external defects. However, internal defects were observed. Specifically, in the 8% of queens enteroliths were observed, which are small stones in the intestinal tract described also in Porporato et al. (2015); in the 9% melanosis of ovary was observed and in 2.7% atrophy of at least one ovary was observed; less than 1% of cases showed empty or dark coloured spermatheca; the most frequent defect observed in the 16% of the queens was abnormal intestinal tract, which appeared brownish/yellowish and swollen.

Mean, standard deviation (SD) and coefficient of variation (CV) of recorded traits are presented in Table I. Number of sperm and volume of spermatheca were very variable, as their CV resulted 82.8 and 41.7%, respectively. Other measures such as tagmata weights, diameter of spermatheca and number of ovarioles were less variable and their CV ranged from 10.1 to 17.9. Concerning body weight, our result was in good agreement with values reported by literature (Tarpy et al., 2010; Delaney et al., 2012; Es'kov & Es'kova, 2013; Hatjina et al., 2014; Porporato et al., 2015). Records from Italian studies were of 186 ± 24 mg average body weight reported by Porporato et al. (2015) and 221 ± 3.09 mg reported by Hatjina et al. (2014). Head and thorax widths were in agreement with the only reports we found published by Tarpy et al. (2010), Delaney et al. (2010) and Hatch et al. (1999). While no references are currently available on abdomen dimensions, we found an average width of the first tergite of 4.8 ± 0.21 mm. Also queen tagmata weights were not found in literature. Our data suggest that the weight of the abdomen was the most variable among the three tagamata and this could be explained by the fact that eggs developmental stages can differ along each ovariole and nutritional state of the queen at the moment of the analysis (filling of the intestinal tract) can also be a source of variation. The length of the right forewing was in agreement with previous reports (Delaney et al. 2010; Es'kov & Es'kova, 2013). Concerning the reproductive organs of the queen,

our result on number of ovarioles was in range considering the 74 ± 14 reported by Proporato et al. (2015) and the 174 reported by Hatijna et al. (2014). Results of spermatheca size and number of sperm in the spermatheca were in agreement with the majority of previously reported studies (Delaney et al., 2010; Tarpy et al., 2012; Hatjina et al., 2014; Porporato et al., 2015).

Table I Number of observation (N), means, standard deviation (SD), and coefficient of variation (CV) for traits measured on queens.

Trait	Unit	N	Mean	SD	CV (%)
Weight Total	mg	147	195.90	19.84	10.1
Weight Head	mg	147	13.22	1.83	13.8
Weight Thorax	mg	147	77.93	8.17	10.5
Weight Abdomen	mg	147	104.76	15.28	14.6
Width Head	mm	147	3.67	0.18	4.9
Width Thorax	mm	147	4.67	0.26	5.6
Width Abdomen	mm	147	4.80	0.21	4.4
Lenght Wing	mm	147	10.07	0.62	6.2
Diameter Spermatheca	mm	147	1.32	0.18	13.6
Volume Spermatheca	µl	147	1.27	0.53	41.7
Number of Ovarioles	n	147	141	25	17.9
Number of Sperms	n million	143	3,6	2,9	82.8

Table II Heritabilities (diagonal), genetic (above diagonal) and phenotypic (below diagonal) correlations estimates for traits measured on queens.

Trait	ww	wh	wt	wa	wih	wit	wia	lw	Ds	vs	o	sp
ww	**0.54**	0.80	0.92	0.84	0.47	0.34	-0.22	0.17	0.23	0.40	-0.13	-0.13
	(0.34)	(0.31)	(0.30)	(0.17)	(0.50)	(0.50)	(1.10)	(0.62)	(0.76)	(0.40)	(0.50)	(0.52)
wh	0.39	**0.51**	0.99	0.61	0.97	0.34	-0.56	0.92	-0.44	-0.44	0.28	0.23
	(0.10)	**(0.35)**	(0.36)	(0.45)	(0.46)	(0.53)	(1.05)	(0.71)	(0.68)	(0.50)	(0.50)	(0.57)
wt	0.67	0.19	**0.50**	0.98	-0.01	0.83	0.54	0.74	0.78	0.61	-0.18	0.15
	(0.06)	(0.11)	**(0.39)**	(0.69)	(0.79)	(0.31)	(0.75)	(0.38)	(0.39)	(0.41)	(0.53)	(0.58)
wa	0.88	0.31	0.29	**0.46**	0.47	-0.14	-0.60	-0.45	-0.24	0.06	-0.15	-0.3
	(0.03)	(0.10)	(0.10)	**(0.34)**	(0.54)	(0.68)	(1.19)	(0.71)	(1.05)	(0.54)	(0.53)	(0.52)
wih	0.36	0.31	0.28	0.27	**0.26**	0.21	0.15	0.96	0.89	0.7	0.55	0.63
	(0.09)	(0.10)	(0.10)	(0.10)	**(0.27)**	(0.67)	(1.08)	(0.48)	(0.48)	(0.52)	(0.58)	(0.56)
wit	0.34	0.30	0.28	0.26	0.32	**0.42**	0.74	0.98	0.59	0.44	0.02	-0.17
	(0.10)	(0.10)	(0.11)	(0.10)	(0.09)	**(0.32)**	(0.36)	(0.31)	(0.57)	(0.43)	(0.53)	(0.56)
wia	0.39	0.17	0.26	0.33	0.25	0.80	**0.13**	0.33	-0.22	0.44	-0.59	0.26
	(0.09)	(0.10)	(0.10)	(0.10)	(0.09)	(0.04)	**(0.26)**	(0.90)	(2.07)	(0.65)	(0.89)	(0.96)
lw	0.34	0.16	0.36	0.20	0.26	0.39	0.30	**0.30**	0.43	0.40	0.79	0.57
	(0.10)	(0.10)	(0.10)	(0.11)	(0.10)	(0.09)	(0.09)	**(0.29)**	(0.81)	(0.50)	(0.59)	(0.52)
ds	0.21	0.04	0.18	0.20	0.08	0.15	0.13	0.10	**0.17**	0.99	-0.42	-0.96
	(0.10)	(0.12)	(0.12)	(0.10)	(0.12)	(0.11)	(0.10)	(0.10)	**(0.34)**	(0.02)	(0.64)	(0.72)
vs	0.22	0.03	0.11	0.18	0.02	0.16	0.16	0.09	0.97	**0.88**	-0.31	-0.70
	(0.13)	(0.13)	(0.12)	(0.13)	(0.12)	(0.13)	(0.12)	(0.12)	(0.01)	**(0.39)**	(0.42)	(0.56)
o	0.01	0.15	-0.003	-0.02	0.08	0.04	0.03	0.02	-0.01	-0.05	**0.70**	0.08
	(0.12)	(0.12)	(0.12)	(0.12)	(0.11)	(0.12)	(0.12)	(0.12)	(0.12)	(0.13)	**(0.35)**	(0.48)
sp	0.03	0.03	0.09	-0.01	0.10	0.04	0.08	0.17	-0.07	-0.07	0.05	**0.57**
	(0.12)	(0.12)	(0.12)	(0.12)	(0.11)	(0.12)	(0.11)	(0.11)	(0.12)	(0.13)	(0.12)	**(0.35)**

ww= body weight; wh= head weight; wt= thorax weight; wa= abdomen weight; wih= head width; wit= thorax width; wia= abdomen width; lw= wing length; ds=spermathecal diamenter; vs= spermatheca volume; o= number of ovarioles; sp= number of sperm in the spermathecal. Standard errors for heritability estimate are reported in brackets.

3.2. Heritabilities, genetic and phenotypic correlations

Genetic parameters are reported in Table II; heritabilities were estimated with univariate analyses of the traits, while genetic correlation were estimated with a bivariate approach. Heritabilities ranged from 0.17 to 0.88 with rather high standard errors due to limited number of individuals. In particular, for body weight and for the weight of individual tagmata heritabilities resulted to be in a narrower range between 0.46 and 0.54. On the other hand, for the width of tagmata the higher estimate was found for thorax width (0.42 ± 0.32), followed by the head width (0.26 ± 0.27) and the lowest was the estimate for the abdomen width (0.13 ± 0.26). Estimate for the length of the right forewing was 0.30 ± 0.29. Estimates for diameter and volume of the spermatheca resulted in 0.17 ± 0.34 and 0.88 ± 0.39 respectively. The latter estimate indicated that high variability of spermathecal volume was mainly due to genetic factors; however, this result should be verified as the volume of spermatheca was approximated to that of a sphere, although this organ may present different shapes (Porporato et al., 2015). The estimate for ovarioles number and for number of sperm contained in the spermathecal were 0.70 ± 0.35 and 0.57 ±0.35, respectively. There are no results in literature on the heritabilities of the traits measured in this study. Overall, the results show considerable genetic variability in the studied population.

Concerning morphological traits (ww, wh, wt, wa, wih, wit, wia, lw), phenotypic correlations ranged from 0.16 to 0.88. Highly correlated traits were body weight with abdomen weight (0.88 ± 0.03) and thorax weight (0.67 ± 0.06); moreover, a correlation of 0.80 ± 0.04 resulted between abdomen and thorax widths. Lower correlations were observed between morphological traits and the length of the right forewing. We observed very low or close to zero phenotypic correlations among reproductive traits (ds, vs, o, sp) except for the correlation between ds and vs (0.97 ± 0.01). The latter is likely explained by the fact that vs is derived by ds using the formula to estimate the volume of a sphere. Reproductive traits seem not to be associated with morphological measures. Our results are in agreement with Corbella and Concalves (1982), Hatch et al. (1999), and Jackson et al. (2011) who

also reported the lack of phenotypic correlation between the body weight of a queen and number of ovarioles. In addition, no phenotypic correlation between number of sperms and spermatheca diameter was found, as previously reported by Jackson et al. (2011).

As expected, our genetic correlations estimates were affected by large standard errors, most probably this was due to the limited number of individuals in our dataset. Therefore, estimates have to be considered with caution. Among morphological traits, weights of tagmata showed high genetic correlations which ranged from 0.61 to 0.99. We found high and positive genetic correlations between weight and width of head (0.97 ± 0.46) and thorax (0.83 ± 0.31) respectively. There was also high genetic correlation between the width of the thorax and the width of the abdomen (0.74 ± 0.34). In addition, we observed high genetic correlations between wing length and wh (0.92 ±0.71), wt (0.74 ± 0.38), wih (0.96 ± 0.48), and wit (0.98 ± 0.31). The high relationship between lw and thorax dimensions may be explained by the fact that wings grow on thorax, the locomotive tagma which groups the legs and the wings. It could be that during the insect development they are tightly regulated. The dimension of the spermatheca positively correlated with wt (0.78 ± 0.39), wih (0.89 ± 0.48). As expected, diameter and volume of the spermatheca correlation was very near to one (0.99 ± 0.02). Positive genetic correlations were found between wing length and number of ovarioles (0.79 ± 0.59) and number of sperms (0.57 ±0.52). The association between wing length and these reproductive traits may be due to the important function of the wings during the mating flight, which depends on wings' movements. The very high negative genetic correlation between the dimension of the spermatheca and the number of sperms it contains was surprising (-0.96 ± 0.72 with ds, -0.70 ± 0.56 with vs), previous reports showed that spermatheca are often not totally filled after mating (Tarpy et al., 2011; Tarpy et al., 2012).

The results on genetic correlations need to be confirmed by additional extensive studies and have to be considered with caution and in this study preference is to rely on the phenotypic correlations as an indicator of the genetic ones.

4. Conclusions

Beekeepers have long selected queens, choosing the "best" on the basis of phenotypic desired features, mainly body size and colour. Queens are responsible for maintaining the balance of the entire colony, therefore their quality is of paramount importance for the beekeeper. The purpose set at the beginning of this study was to perform a genetic analysis in order to obtain estimates of the heritability of a series of traits that, which among many others, could be useful to for the evaluation of the quality of a queen. Statistical analysis confirmed the existence of correlations rather intuitive between some morphological measures. Other results shed light on aspects which were counterintuitive in principle, *i.e.* the absence of phenotypic correlation between body size and ovarioles number. Heritability (h^2) is a parameter that generally describes how easily parents transmit to their offspring a determined phenotype and which, on the other hand, has practical operational utility for selection (the value of h^2 is directly proportional to the success of selection as it explains what part of the phenotypic variability is attributable to the genetic one). The heritability of total body weight, spermatheca volume, number of ovarioles, and number of stored sperm looks promising. However, it would be interesting to reproduce the analysis on a larger number of individuals, to confirm or change the indicative conclusions from this study.

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Authors contribution

EF, RR, GP, DL and MP conceived and planned the experiments. GP, RR and FP contributed in the supervision of the project. EF implemented and carried out experiments during 2017, FT performed semen analysis. EF organized data collection in 2018. EF, RR and GP contributed to the analysis and interpretation of the results.

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Chapter 4

Honey bee pupal length assessed by CT-scan technique: effects of *Varroa destructor* infestation, developmental stage and spatial position within the brood comb

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Honey bee pupal length assessed by CT-scan technique

Abstract

Honey bee pupae morphology can be affected by a number of stressor, but in vivo investigation is difficult. A computed tomography (CT) technique was applied to visualize the brood comb inner structure without damaging the brood. The CT scans were performed on a brood comb containing pupae developed from eggs laid by the queen during a time window of 48 hours. From each CT scan, the position of each pupa was determined by recording coordinates to a common reference point. Afterwards, every brood cell was inspected in order to assess the developmental stage of the pupa, the presence of *Varroa destructor*, the number and progeny of foundress mites. Using data on 651 pupae, the relationships between varroa infestation status, developmental stage and spatial position of the pupa within the brood comb, and its length were investigated. Pupae at 8 post-capping days were shorter than pupae at 7 post-capping days. Pupae in infected cells were significantly shorter than those in varroa-free cells and this effect was linked both to mite number and stage and to the position in the comb. Overall, the results suggest that the CT-scan may represent a suitable non-invasive tool to investigate the morphology and developing status of honey bee brood.

1. Introduction

In recent years, honey bee colony losses have been recorded throughout Europe and the World (Oldroyd et al., 2007; vanEngelsdorp et al; 2007; Neumann and Carreck, 2010). While a multitude of causative factors for this phenomenon have been extensively debated, now infestation with the invasive ectoparasitic mite *Varroa destructor* is considered one of the most significant causes for colony losses (Anderson and Trueman, 2002). The mites depend on honey bee brood for reproduction, and the reproductive cycles of host and parasite are tightly linked to each other (Rosenkranz et al., 2010). Within the isolated and protected environment of a capped cell, the reproducing mites and their offspring feed on the developing honey bee pupae. While the native host *Apis cerana* has evolved a multitude of behavioural adaptations to limit the damages inflicted by the parasite, heavy mite infestation in colonies of *A. mellifera* causes severe damage, typically associated with secondary virus infections and a complex of symptoms known as varroosis, and will eventually lead to colony collapse. At honey bee individual level, it was reported that varroa infestation causes weight loss and reduced life span (De Jong et al., 1982; Schneider and Drescher, 1987; Colin et al., 1999; Bowen-Walker and Gun, 2001). Moreover, it was reported that multiple infestation of mites in one cell can cause shrinkage of the bee abdomen and increases the incidence of deformed wings (Ritter and Akratanakul, 2006).

The alteration of honey bee pupae morphology including size and length can be considered of value to assess the negative effects of mite infestation of the colony (De Jong et al., 1982; Schneider and Drescher, 1987; Colin et al., 1999; Bowen-Walker and Gun, 2001). Current methods for varroa load assessment in the brood, as for instance opening a random sample of capped brood cells (n=200) and measuring the percentage of infested cells, are invasive, partially or totally destructive and time consuming (Dietemann et al., 2013). For research and field purpose it is important to develop innovative and non-invasive methods to assess the brood mite infestation degree of a colony. Among the currently available imaging diagnostic techniques, computed tomography (CT) imaging technique

employs x-rays to produce cross-sectional images (slices) of a scanned object, allowing the visualization of its inner structures without inherent damages to live tissues and materials. In this study, a non-destructive CT technique and image analysis approach coupled with brood manual inspection was used to clarify the relationship between varroa mite infestation status and pupa length, taking into consideration other factor such as the spatial position of the pupa within the comb and its developmental stage. Also the distribution of infected cells throughout the brood area of the comb was investigated.

2. Material and methods

The experiment was carried out in June 2018 at the Faculty of Veterinary Medicine, University of Milano, Via dell'Università n. 6, Lodi, Italy. Pupae from one brood comb were analysed. The brood comb came from a honey bee colony in good health status and headed by naturally mated queen. At the beginning of the experiment, the queen was caged on an empty comb and released after 48 h. This procedure permitted to obtain a comb hosting eggs within a range of maximum two days' age difference. After queen release, the brood comb was put back into the colony to allow the further development of brood under natural condition. The CT scan was performed after 14 days from the beginning of the experiment to obtain a population of pupae aged between stages 7-days and 8-days after capping (Büchler et al., 2017). The comb was submitted to CT examination and images were acquired with a 16-slices CT scanner (GE Brightspeed®, GE Healthcare Milano – Italy), using a high resolution filter. Scanning parameters were set as follows: kV = 120, mA = 250, slice thickness = 0.625 mm, pitch = 0.9375. After CT scans acquisition, the comb was stored at -20°C until manual inspection.

2.1. Image analysis

CT scans were visualized with image viewer Weasis (version 2.0.5), a free software which permits to handle DICOM files (Digital Imaging and COmmunications in Medicine). A collection of images out of 1452 scans were selected (approx. one image every 0.5 cm) in order to obtain a "library" showing the content of every cell row in its centre, along the coronal plane of the comb where its original position within the hive was taken as the reference and the entrance of hive represented the front (Figure 1, right). For each scan, the exact coordinates of each capped cell in the comb were extracted using Weasis (version 2.0.5). For each selected image, the position of each pupa was determined by means of the coordinates to a common reference point (i.e., the top left part of the

comb). Moreover, the length of each pupa was assessed using the measuring tool provided by the program viewer.

2.2.Comb inspection

In order to assess the developmental stage of the pupa and the infestation of varroa mite, each cell of the comb was individually and manually inspected. The wax cap of each cell was opened with a scalpel and the pupa was extracted using a pair of tweezers. The age of the pupae and the presence of varroa mites were recorded according to Büchler et al. (2017). In addition, when mites were found, also the number of foundress mites (i.e., adult females with offspring) and the number of progeny were recorded.

2.3.Statistical analysis

For the analysis of the length of the developing honey bee pupae, different factors were considered. Firstly, the position of pupae within the brood was taken into account by sub-setting the brood area of both sides of the analysed comb into 12 uniform squares by a grid containing 12 sections (3 rows by 4 columns, see Figure 1, left). Secondly, the age of the pupae within each cell was considered as a variation factor. Lastly, the effect on the length of the pupae given by the presence of varroa in the cell was tested considering three different categories: i. Mite presence or absence; ii. Number of foundress mites; and iii. Total number of mite's individuals found in the cell.

To test the relationship between the presence of varroa and the position of the cells within the 12 sections of the brood comb, a χ^2 analysis was performed. This permitted to assess if varroa mite was distributed in a uniform way within the brood comb.

We chose to analyse the length of pupae situated in the two central sections of the comb assuming that such area shares a slightly higher and more constant temperature, which can influence the size of

the developing insect (Büns and Ratte, 1991; Sibly and Atkinson, 1994; Petz et al., 2004; Becher and Moritz, 2009).

The following fixed model was fitted to data, using PROC GLM of SAS®:

$$y_{ijkl} = \mu + S_i + A_j + V_k + e_{ijkl}$$

where μ is the overall mean oh the length of the pupa, S refers to i^{th} section of brood in the comb (i = 1,2), A is the j^{th} developmental age of the pupae (j = 1,2), V is the k^{th} effect of varroa in the cell, and e is the random error term of the l^{th} observation (l=1, 651).

As regards to the effect of varroa, firstly V was fitted as a binary factor indicating the presence or absence of Varroa within the cell (k = 0,1). Secondly the number of foundress mites was considered, where V term varied between 0, 1 foundress mite and more than one founder (k = 1,3). Lastly, the effect of the total number of mites within the cell (foundress, son and daughters) was assessed considering V ranging from 0 to 5 individuals (k = 1, 6), where cells with 2 mites were pooled with cells with 1 mite and cells with more than 5 mites where pooled with cells with 5 individuals.

Least square (LS) means were separated by pair-wise t test and Bonferroni adjustment was applied. Mean separation for main effects were performed on least square mean using PDIFF option of SAS®. Statistical differences were declared at $P < 0.05$.

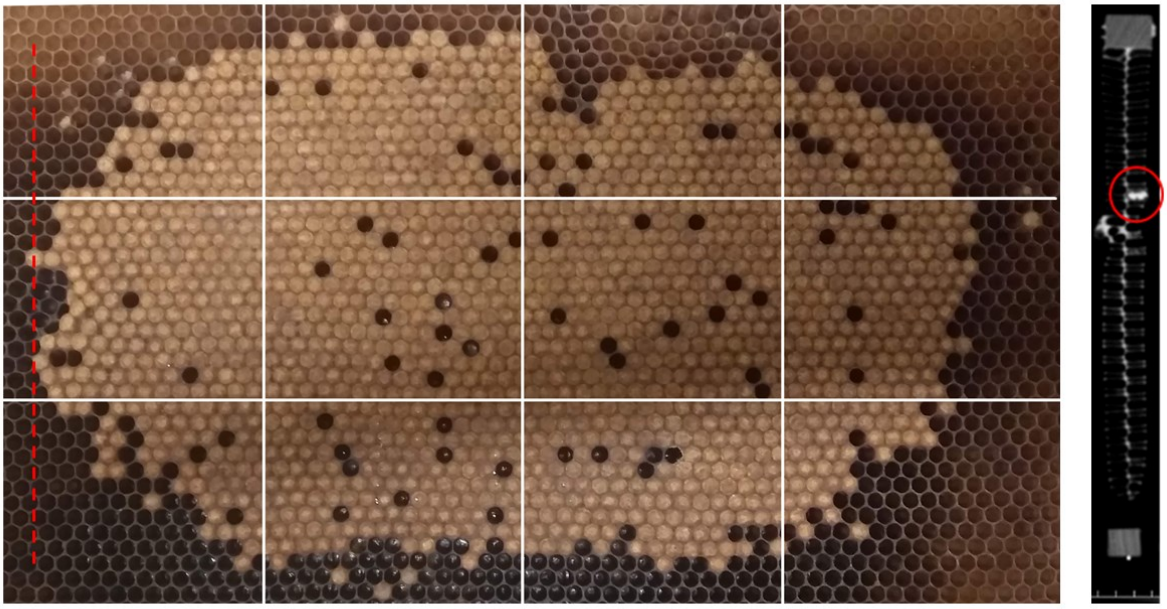

Figure 1. Sections of the brood and CT-scan of brood-comb structure. Left: one side of the analysed comb. The red dashed line shows the position of the vertical cell row shown by the scan image on the right. Right: CT-scan of the analysed comb (WW 1200, WL -700). The red circle indicates the pupae in the capped cell in correspondence to the red dashed line on the left.

3. Results and discussion

A total of 2466 pupae were inspected for presence of varroa mite in their cells and the corresponding lengths were measured from the CT images collection. One-hundred two out of 2466 cells were infested by the mite, corresponding to a 4.1% total true brood infestation of the analysed comb. The distribution of observed and expected varroa within each section is reported in Figure 2. The association between presence and absence of varroa and the position of the cells in the twelve sections was statistically significant ($\chi^2 = 75.41$, DF = 11, $P < 0.001$). Moreover, considering the distribution of the presence of varroa within each section, the two central ones showed more varroa mites than expected (section 6: $\chi^2 = 39.95$, DF = 1, $P < 0.001$; section 7: $\chi^2 = 4.49$, DF = 1, $P = 0.03$). Besides, less mites than expected were observed in sections 8 ($\chi^2 = 5.30$, DF = 1, $P = 0.02$), 9 ($\chi^2 = 4.04$, DF = 1, $P = 0.04$), and 10 ($\chi^2 = 7.85$, DF = 1, $P < 0.01$). These results showed that varroa mites preferentially invaded cells in the inner brood area of the analysed comb rather than infesting evenly the brood cells. This could be an indicator of preference of varroa mites for central brood areas, where temperatures are known to be kept slightly higher and more constant by worker honey bees compared to the periphery of the combs (Becher and Moritz, 2009).

The two central sections contained 651 cells whose 58 were parasitized resulting in a partial brood infestation of 8.9%. This value of brood infestation was higher compared to total brood infestation rate reported above (4.1%). This results confirmed the importance of the random sampling of cells when the manual brood inspection is applied for estimating the brood mite infestation.

Results from each of the three statistical models showed that the stage of the pupae, the position in the brood area (i.e. the two central squares analysed) as well as varroa mites had significant effects on the length of the pupae ($P < 0.001$). Each model showed that pupae at stage 8 were significantly shorter than pupae at stage 7. Statistically significant difference was also found between the length of pupae in square 6 and square 7. The pupae analysed in square 6 were longer than pupae in square 7; this result could be explained by the fact that square 6 was facing the entrance of the hive, which

was orientated to South and probably exposed to higher temperatures.Table *I* reports the Least Square (LS) means of the length of the bee pupae estimated with each of the three models considering the variable varroa in the three separated categories described above. LS means from the first model showed that the presence of varroa significantly affected the length of the pupal stage by a reduction of 0.35 mm (from 10.54 mm to 10.19 mm) which represents approx. the 3% of the average varroa mite free pupa length in our sample. In the second model the effect of varroa was considered as the number of foundress mites found in the analysed cells. LS means for the length of pupae hosting one foundress mite was 10.20 mm and was significantly shorter compared to varroa free pupae (10.54 mm). The length of pupae parasitized by two or more foundress mites was 10.08 mm and significantly shorter than varroa free pupae, but not significantly shorter than pupae with one foundress mite. Results from the third fitted model showed that the length of the pupae was significantly shortened also by the presence of more than three individuals within the same cells. It is possible to see a trend in the length of the pupa which became shorter the more mite individuals were present in a cell. It could be supposed that such a reduction in length could be due to the nutritional behaviour of the mites on the developing honey bees. Indeed, varroa mites during their reproductive stage within the brood cell pierce the cuticle and feed on honey bee's internal tissues (Rosenkranz et al., 2010). Considering that the size of the pupae can be correlated with its weight, the above results agree with previous studies focused on the effect of parasitization on the weight of honey bees at their emergence (De Jong et al., 1982; Schneider and Drescher, 1987, Bowen-Walker and Gun, 2001).

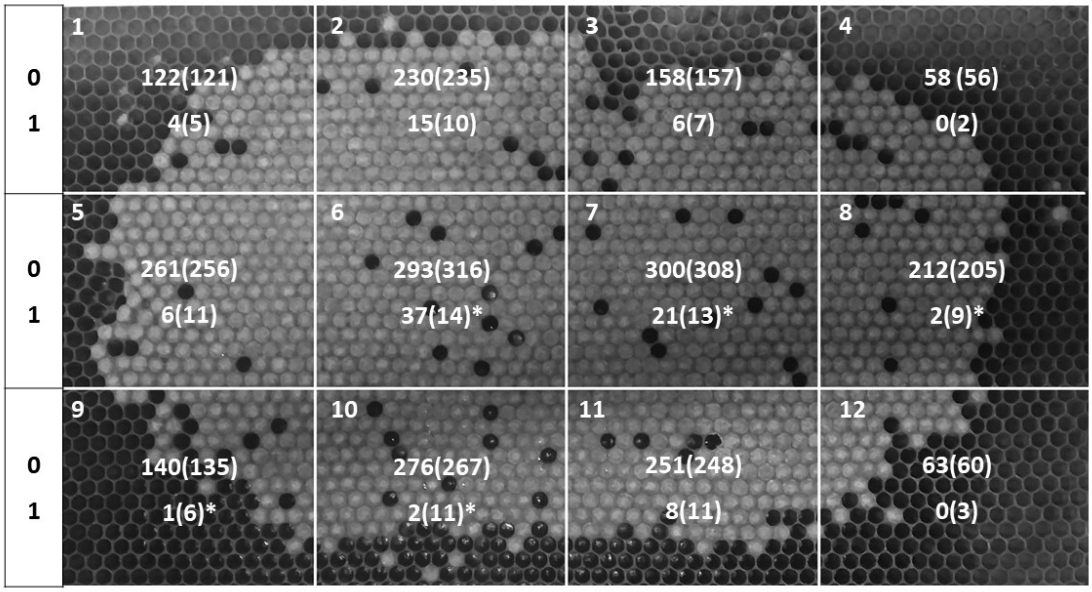

Figure 2 Brood area sections (1 – 12) and superimposed contingency table for absence (0) and presence (1) of observed and expected (in brackets) varroa mites. * Significant χ^2values ($P < 0.05$).

Table I LS Means and standard errors (SE) and relative number of observations (N) of length of pupa for the three categories of mite infestation: presence or absence; number of foundress mites; total number of mites.

Presence/absence model	N	LSMeans ± SE
0 – absence	593	10.54 ± 0.02^{a}
1 - presence	58	10.19 ± 0.04^{b}
Number of foundress mites model		
0 - absence	593	10.54 ± 0.02^{a}
1 foundress mite	52	10.20 ± 0.04^{b}
>=2 foundress mites	6	10.08 ± 0.11^{b}
Total number of mites model		
0 – absence	593	10.54 ± 0.02^{a}
1 or 2 mites	9	10.30 ± 0.09^{ab}
3 mites	17	10.24 ± 0.07^{b}
4 mites	18	10.16 ± 0.06^{b}
≥5 mites	14	10.09 ± 0.07^{b}

Means with different superscript are statistically different ($P < 0.05$)

4. Conclusions

To our knowledge, this is the first report analysing the length of developing pupae within intact brood using CT-scan technology. This would be a relevant new tool to allow morphological measurements of honey bee's pupal stages without uncapping the cells during in vivo studies. Our findings showed that the length of the pupae was influenced by the developmental stage, the position within the brood comb area and the parasite load. The length of pupae at stage 7 and stage 8 (post-capping days) is negatively affected by the presence of the mite, and this reduction could be proportional to the number of individuals present in the cell. Noteworthy, varroa mites seem to invade central brood areas where temperature was reported to be slightly higher and more stable, rather than evenly invading brood cells.

Commonly, CT scans prices are usually high in veterinary medicine due to running costs, hardware maintenance and anaesthesia protocols, but CT-scans' prices have fallen significantly over the past few years since their application is spreading from clinical settings to animal production systems. It is also worth remembering that unlike what happens in the current clinical practice, for honey bee colonies the instrument could host simultaneously up to 36 combs/scan, thus allowing the monitoring of several colonies by one scan.

From the perspective point of view, our study suggest that CT-imaging could become a fast and non-destructive approach to explore the developing status of the honey bee brood stages.

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Chapter 5

Study on honey production in a small breeding populations in Italy

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Manuscript in preparation

Honey yield genetic parameters

Abstract

Honey bees represent an important productive livestock species due to hive products such as honey, bee wax, pollen and to its invaluable role as pollinators of agricultural crops and wild flora. The estimation of breeding values in genetic selection programmes is a tool of paramount importance, as it allows to identify genetically superior individuals that can then become parents of the next generation, improving traits of economic interest. Genetic evaluation in the honey bee is special due to their peculiar mode of reproduction. In fact, ordinary rules for the estimation of genetic relationships are not applicable to honey bees due to queen's multiple mating and haploid males producing a multitude of identical sperm cells.

The aim of this work was to estimate variance components and genetic parameters for honey yield for a small testing population of honey bees in Northern Italy. Honey production data was collected for three years consecutively, from 2016 to 2018, three times a year on three different testing groups of honey bees. Estimated heritability for the total yearly honey production of a colony was 0.14 (0.12). Meanwhile, considering separated productions within a year, the estimate for repeatability of honey production of a colony within the same year was in 0.53 (0.04).

1. Introduction

The Western honey bee (*A. mellifera*) is an insect that is bred worldwide especially for honey production and pollination services (Klein et al., 2007). Worker bees, after hatching, spend the first part of their life inside the hive taking care of brood, comb, feeding their queen and colony thermoregulation, and during the second part of their life they become foragers and take care of supplies for their colony (Winston, 1987). In fact, when the weather is good, foragers spend the daylight flying outside the hive to find and collect water, pollen and nectar. Nectar and pollen are plant-produced substances which fulfil the nutritional requirement of brood and adults of the colony. Honey is produced from nectar or honeydew and its production depends on many factors. First, the colony has to be in good health condition and have a large number of foraging bees. Another conditioning factor is the presence of abundant nectar sources accompanied by favorable weather: both dry and very rainy periods are detrimental (Sammataro & Avitabile, 2011). Drought in fact can reduce or even stop the production of nectar by the plants, and the rain can spoil flowers and doesn't allow the bees to easily fly and forage. Beside environmental factors and colony general condition, also beekeeping practices can substantially affect honey production (Lodesani, 2003). In modern beekeeping, honey is harvested from supers, which are special additional boxes positioned over the hive on occurrence, separated by a queen excluder, a grid which avoids the queen to lay eggs where honey is stored (Lodesani, 2003).

Beside disease resistance and gentleness, honey production has always represented a central goal in breeding programs. The estimation of breeding values in genetic selection programmes allows to identify genetically superior individuals that can then become parents of the next generation, improving traits of economic interest. Genetic evaluation in the honey bee is peculiar due to their mode of reproduction. In fact, ordinary rules for the estimation of genetic relationship are not applicable to honey bees due to queen's multiple mating and haploid males producing a multitude of identical sperm cells.

Thanks to recent advances in statistical method, nowadays it is possible to evaluate testing population exploiting BLUP animal model. This statistical method, successfully used for other livestock species, was tricky to apply to honey bees because of the peculiarity in the relationships between the individuals of the colony. The maternal side is direct and equal to mammal kinship. Queens mate with multiple drones at the beginning of their life and store the sperm in the spermatheca. Therefore, workers in a colony descend from several haploid males, on average ca. 12 drones (Tarpy et al., 2004). The most advanced method for the calculation of relationship matrix and its invers was published by Brascamp and Bijma (2014), which improved the method by Bienefeld et al. (2007). The improved method better captures the relationships between half-sib and full-sib colonies, which was averaged in Bienefeld et al. (2007).

The aim of this study was to assess heritability for honey production in a small breeding population in Northern Italy using data collected during three years, from 2016 to 2018, applying the method of Brascamp and Bijma (2014). Moreover, we explored the repeatability of honey production of a colony within year, which has never been investigated before.

2. Material and methods

2.1. Colonies and data collection

Honey production data was collected during productive seasons 2016, 2017, and 2018. Colonies enrolled for this study were kindly made available from Melyos, an Italian bee breeding and beekeeping company who set up and managed the testing populations. The first group of colonies (n=143) was distributed in 4 apiaries nearby Lodi (Lombardy Region, Italy) and was evaluated in 2016. The second group (n=72) was distributed in two of the four apiaries of 2016 and was evaluated in 2017. In 2018, the F1 from 2016 population (n=104) was evaluated in a fifth different apiary located in Lesmo (Milano Province, Lombardy Region, Italy) ca. 30 Km north from Lodi. Every year, colonies were headed by groups of sister queens mated with one paternal line, which was different for each year. For every queen, pedigree information was available. In each year, tested colonies were set up the previous autumn by inserting the queens in queenless nuclei. Starting from autumn, colonies under test were kept following the most standardise way during the next year. When feeding was necessary, every colony was supplied with the same amount of feed. Moreover, the brood in the testing population was managed with the "tight nest" (Seifried and Seifried, 2011). This method allows the bees in the hive only the room needed for the brood according to their queen's laying eggs ability, leaving only one lateral comb for nest honey storage. This nest managing system is adopted by professional beekeepers oriented to maximize honey production. Therefore, worker bees are induced to store collected nectar in the supers, where the beekeeper can harvest it.

2.2. Honey harvesting

Honey production data was collected three times each year, in correspondence to the three main blooming period in the region. The first honey production is characterized by dandelion (*Taraxacum officinale*) spring blooming and it is harvested in April. The second and main honey production is

represented by the *Robinia pseaudoacacia* honey, and it is harvested in May. The third production is characterized by multifloral blooming in June (mainly Linden, *Tilia tomentosa*).

Supers were weighted before and after each honey harvest. Each single net production was represented by the three honey harvest periods, meanwhile the total yearly honey production was obtained by summing the three single net honey productions for each colony.

2.3. Statistical analysis

For the analysis, estimates of the genetic relationships between groups of workers and queen in colonies are required, as well as those with the groups of drone producing queens with which queens were mated. We used the method of Brascamp and Bijma (2014) to estimate these relationships. The pedigree file was built according the procedure described in Brascamp et al. (2016). To estimate variance components, heritability and repeatability, the statistical package ASReml and the pin function of the nadiv package were used in the computing environment R (Butler, 2009; Wolak, 2012; R Core Team, 2015). Only the genetic effect of the workers was included, as the number of records and individuals did not allow us to simultaneously estimate the queen and the worker effects and their covariance. Following Brascamp et al. (2018), we used the average additive genetic variance of worker groups to calculate phenotypic variance, assuming that all worker groups in a colony contribute to the honey yield. The heritability then is the proportion of the phenotypic variance that is due to this average additive genetic variance.

First we analysed the overall sum of honey production using the following mixed model:

$$y_{ij} = \mu + ApY_{ij} + A_{w\,ji} + e_{ij}$$

where μ represents the overall mean, ApY is the fixed effect of the combination of the ith apiary (i = 1, 5) and j^{th} year (j = 1, 3); A_w represents the random genetic effect of the j^{th} colony where the number of colonies per apiary per year varies (Table I), in total (j = 1, 319), and e represents the random error

term. This model allowed us to estimate the heritability of the sum of produced honey by a colony in one year.

Secondly, we fitted a repeatability animal model:

$$y_{ijkl} = \mu + ApTY_{ijk} + A_{w\,lkij} + pe_l + e_{ijkm}$$

where μ represents the overall mean, *ApTY* is the fixed effect term representing the combination of the i^{th} apiary (i = 1, 5), j^{th} harvest time (j = 1, 3) and k^{th} year of observation (k = 1,3); A_w represents the random genetic effect the l^{th} colony (319 colonies in total), *pe* represents the random permanent environmental effect the l^{th} colony (l = 1, 830), and *e* represents the random error term. This model allowed us to estimate both heritability and repeatability for single honey harvest of a colony in one year.

Table I Distribution of colonies among the apiaries during the three testing years

Year/Apiary	A1	A2	A3	A4	A5
2016	37	35	35	36	-
2017	49	-	23	-	-
2018	-	-	-	-	104

3. Results and discussion

The composition of the colonies per apiary and the testing location varied because they depended on the breeder's colonies and apiaries availabilities. Means and standard deviation for each production in each year are shown in Table II.

From data in Table II, it appeared that honey harvest in the last three years did not followed the usual trend. In the region where honey was produced (Lombardy, Northern Italy) usually the main production is the second harvest of the year during *R. pseudoacacia* blooming. This was observed during 2016 and 2018. The second important harvest should be the third (Linden), and this only was observed in 2018. In 2016, the third production was spoiled by suboptimal meteorological conditions. The year 2017 was an anomalous year for honey production in Northern Italy. It was characterized by a good first harvest, a very poor second harvest and a modest third harvest. The second harvest of 2017 was also undermined by unfavorable weather conditions. The only expected honey production trend was observed in 2018.

Table III shows the estimates for the variance components and the resulting heritabilities and repeatability of honey harvest both considered as yearly total harvest (sum model) and considering separated productions within a year (repeatability model). Therefore, it is important to consider that the two honey yields differed between both model. In the sum model, the variance components referred to the yearly sum production of a colony, while in the repeatability model, the dependent variable was represented by each of the three net weights of honey produced by colonies during the three harvest times within a year. Genetic parameters estimated with BLUP Animal Models for the total yearly production can be found in the literature (e.g. Bienefeld and Pirchner, 1990; Brascamp and Bijma, 2016). Heritability for the sum of productions was 0.14 (0.12). This value was lower than the half of previously reported heritability of 0.42 (0.08) by Brascamp et al (2018) and it also lower than the 0.26 (0.10) reported by Bienefeld and Pirchner (1990). The lower heritability estimate could be explained by both lower genetic variance for this trait in our testing population. Compared to the

value reported by Bienefeld and Pirchner (1990) our lower estimate could be explained by the correction of the additive genetic variance for group of workers as described by Brascamp et al. (2018). Heritability estimated with the repeatability model was even lower (0.11 ± 0.08). But interestingly, the repeatability of honey production of a colony between two harvests within the year of production was medium-high (0.53 ± 0.04). In fact, the estimated permanent environmental variance was fairly high compared to the additive variance component (Table III). Repeatability (r) is the correlation between repeated records on the same colony within year (Falconer and Mackay, 1996). Our results indicated that honey productions of a colony within a year are affected by permanent environmental effects which influence the similarity between two harvests of a colony. In this particular case, a permanent environmental effect could be the presence of the queen. But additional research is needed on extended datasets in order to fit the genetic effect of the queen in the repeatability model to improve the estimates of variance components. Studies in which honey heritability production was investigated fitting the genetic effects of the queen resulted with negative genetic correlation between the genetic effect of workers and queens (Bienefeld and Pirchner, 1990; Brascamp and Bijma, 2016). This aspect is considerably slowing down the genetic improvement of traits of interest like honey yield.

Table II Means and standard deviations in brackets are reported for the three honey harvests within a year and for the overall sum of yearly production.

Year	First harvest	Second harvest	Third harvest	Sum of Harvests
2016	6.31 (3.78)	13.36 (5.21)	1.38 (1.12)	17.84 (9.69)
2017	9.53 (4.72)	5.23 (2.42)	6.75 (3.35)	19.27 (10.35)
2018	4.12 (3.05)	15.36 (5.89)	7.07 (3.73)	25.23 (11.77)

Table III Estimated genetic parameters for honey production. Variances (*Var*) of genetic effect for the average of workers ($\overline{w}$), permanent environmental effect (*pe*), residual (*e*) and phenotypic (*P*). Derived from these are estimates of heritability (h^2) and repeatability (*r*). Approximate standard errors are reported in brackets.

	Sum model	*Repeatability model*
$Var\ (\overline{w})$	15.2 (13.1)	1.7 (1.4)
$Var\ (pe)$	-	6.7 (1.3)
$Var\ (e)$	90.8 (12.1)	7.4 (0.4)
$Var\ (P)$	106.0 (9.1)	15.8 (1.0)
h^2	0.14 (0.12)	0.11 (0.08)
r	-	0.53 (0.04)

4. Conclusions

The results of this study confirmed the strong environmental influence for such a complex trait as honey production. Heritability was 0.14 when total honey yield was considered, while the estimate was 0.11 considering each harvest individually. Within one year, honey production showed to be influenced by fairly strong permanent environmental effects, which might be caused by the presence of the same queen in the whole harvest period. Future analyses could focus on fitting the effects such harvest time/ apiary / year as random effects, considering that the beekeeper has only partial control on environmental factors, as intended for other livestock. In fact, they could be considered as common environmental effects. Additional analyses are required to better understand these aspects.

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General conclusions

First of all is noteworthy to consider that dealing with honey bees implies a fine scheduling of time and work, due to their biological cycle. At this regards, all the field work was accomplished during springs and summers, when honey bees were active; meanwhile molecular research and data analysis was concentrated during the winter period (and rainy days).

This three years' research study was aimed to establish suitable and rational collection of phenotypes and to explore the analysis of various datasets to estimate genetic parameters like heritabilities. The work was characterized by a transversal approach: from in-field phenotypic data collection to SNP discovery. Overall, this thesis' work focussed on important and different phenotypes for modern beekeeping such as hygienic behaviour, quality of the queens, potential new approaches for in-vivo studies and honey production and it represents an example on how to measure and analyse traits related to modern beekeeping practices.

In the first chapter we demonstrated that FKB* requires less time and liquid nitrogen, and has a smaller measurement error, resulting in higher repeatability. Results showed that to accurately phenotype a colony for HB, any of the two tests should be repeated at least twice during active season of honey bees and that heritability for the average HB score indicates good prospects for genetic improvement of HB. Overall, this chapter presented a methodology which can be used by those honey bee breeders or geneticists who wants to improve brood disease resistance in their population.

In the second chapter we presented a methodology to obtain DNA from wings cuttings of queens. Results from this chapter open the door to honey bee genomics for bee breeding. The proposed approach permits to collect genetic information from queen bees very early in the season. Therefore, it can be useful for the genotyping and reducing the generational interval in future breeding programmes. Further work will analyse possible association between genetic variability and

phenotypes of interest and to investigate the effects of the discovered variants and their association with HB.

The third chapter aimed to obtain preliminary estimates of the heritability of a series of traits that, among many others, could be useful to for the evaluation of the queen's quality. Statistical analysis confirmed the existence of correlations rather intuitive between some morphological measures. Other results shed light on aspects which were counterintuitive in principle, i.e. the absence of phenotypic correlation between body size and ovarioles number. It would be of interest to reproduce the analysis on a larger number of individuals, to confirm or change the indicative conclusions from this study. Most of all to study potential association between queen characteristics and colony performances. Beside the intrinsic limits of the study arising from the small sample size, this chapter sets a cornerstone for future breeding programs focussing on the most important individual of a colony, the queen, rather than colony related performances. Moreover, it provides instructions for collecting phenotypes and analysing data with animal models, the most powerful statistical approach in animal breeding and genetics.

The fourth chapter is a cornerstone study which shed light on pupal morphology, analyzing the length of pupae within intact brood using CT-scan technology. This chapter is the result of a project that I independently thought and set up. Our finding showed that the length of the pupae was influenced by the developmental stage, the position within the brood comb area and the parasite load. Noteworthy, the distribution of *V. destructor* within the comb seems to be unevenly concentrated in the central part of the brood comb, where temperature is maintained slightly higher and more constant by worker honey bees. CT-imaging could become a fast and non-lethal approach to assess the developing status of honey bee brood provided that a suitable post processing image analysis will be implemented. It could also represent a valid tool to follow up colonies in the assessment of any potential negative outcomes of pharmaceuticals use on the capped brood, which is a parameter that it is difficult to monitor in field conditions.

Lastly, during the last three years I collected data on honey production, which is one of the major breeding goal for beekeepers in Italy. Results of the fifth chapter confirmed the strong environmental influence for honey production. Within one year, honey production showed to be influenced by fairly strong permanent environmental effects. This preliminary approach shed light on the need to adapt different strategies to the analysis of such complex trait ad they could focus on considering to include in the model common environmental effects such as harvest time/apiary/year. Additional analyses are required to better understand these aspects.

From this thesis, innovative insights can be found and further be elaborated for including new and powerful aspects for the genetic improvement of honey bees, not only for colony level traits such as disease resistance but also shifting the attention to an individual level where important traits for queens can be improved.

In conclusion, the general result of this thesis is represented by the effort in providing a solid contribution and new knowledge to future bee breeders and geneticists.

PhD candidate activities from 1st October 2016 to 30th September 2018

Attendance to courses

- **"Genetic Improvement of Livestock"** at Wageningen University and Research. From Nov-Dec 2017. ECTS 6.00. Final mark 8/10 approved.
- **"Animal Breeding and Genetics"** at Wageningen University and Research. Sept-Oct 2017. ECTS 6.00 Final exam 10/10 approved.
- **"Communication 2"** held by Dr. Ettore Galanti. 3,23 march 2016, 14 April 2017. Final exam: approved. 12 hours.
- **"Medical statistic 2"** held by Dr. Elisabetta Sala. From 1st February to 4th April 2017. Final exam: approved.
- **"Advanced Statistic for Animal Science"** held by ASPA- Associazione per la Scienza e le Produzioni Animali, 5-10 Febraury 2017
- **"Statistic"** held by Dr. Roberto Ambrosini. 5-28 September 2016. 24 hours.
- **"Genomics of pathology"** held by Dr. Giulietta Minozzi. 7,15,23,28 June 2016. Final exam: approved. 17 hours.
- **"Digital imaging and image integrity in scientific publication"** held by Dr. Valentina Lodde. 9, 11-13 May 2016. Final exam: approved. 17 hours.
- **"Corso di introduzione all'analisi sensoriale del Miele"**. Associazione Ambasciatori del Miele. Milano. 28th-29 th May and 4 th -5 th June 2016. 36 hours.
- **"Medical statistic 1"** held by Dr. Elisabetta Sala. From 1st February to 4th April 2016. Final exam: approved. 30 hours.
- **"Communication 1"** held by Dr. Ettore Galanti. 3,23 march 2016, 14 April 2016. Final exam: approved. 12 hours.

Attendance to seminars, workshops and scientific meetings

- **"Canine Genomics and Veterinary: from domestication to pathologies"** University of Milan, June 18 2018.
- **"Ambiente, Apicoltura e Agricoltura"** Associazione Forestale del Trentino. 9th March 2018, Trento, Italy
- **"Giornata di Approfondimento su ricerca e selecione di specifici caratteri genetici negli USA"** held by Marla Spivak and Jackie Park-Burris, 34° Congresso AAPI. 31 Jenuary- 4 February 2018. Paestum, (NA), Italy.
- **"Autoformazione autunnale 2016".** AIAAR- Associazione Italiana Allevatori Api Regine. Rimini (RN). 15th-16th November 2016.
- "**General Linear Model and Experimental Design using R**" held by Prof. Rocco Micciolo from Dipartimento di Psicologia e Scienze Cognitive Università di Trento. At Laboratorio di Statistica Medica, Biometria ed Epidemiolgia "G. A. Maccacaro", Milano. From 14th to 18 th march 2016. 45 hours.
- **"Le pesti delle api: attualità e prospettive".** Dipartimento di Scienze Veterinarie e Sanità Pubblica. Università degli Studi di Milano. 26th february 2016. 4 hours.
- **"Autoformazione autunnale 2015".** AIAAR- Associazione Italiana Allevatori Api Regine. Castel San Pietro Terme (BO). 10 th-11 th december 2015. 18 hours.
- **"Ripartire dalle api. Strategie di collaborazione ambientale".** Fondazione Edmund Mach. San Michele all'Adige (TN). 13th november 2015. 9,30 hours.

National Conferences

- **Association for Animal Science and Production** - XXII ASPA Congress, June 13rd to 16th 2017, Perugia (PG), Italy.
- **Società Italiana di Parassitologia** – *XXX SOIPA Congress, June 26th to 29th 2018, Milano (MI), Italy.*

International Conferences:

- **"The 7th European Conference of Apidology"** EURBEE- European Association for Bee Research. Cluj-Napoca, Romania. 7 th-9 th September 2016.
- **"12 th COLOSS Conference"**. COLOSS- Prevention of Honey bee Colony Losses. Cluj-Napoca, Romania. 10 th -11 th September 2016.
- **"13 th COLOSS Conference"**. COLOSS- Prevention of Honey bee Colony Losses. Athens, Greece. 2nd – 3rd November 2017.

Awards

- Winner of the Best Student Poster at 13th Coloss Conference. 2-3 November 2017, Athens, Greece.

Visiting Student

- **National Research Council.** Institute of Agricultural Biology and Biotechnology. From March 2018 to September 2018. Supervisor: Dr Stefania Chessa.
- **WUR.** Wageningen University and Research. Animal Breeding and Genomics group. From September 2017 to end of January 2018. Supervisors E. W. Brascamp and P. Bijma.
- **DISAFA.** Università di Torino. Dipartimento di Scienze Agrarie, Forestali e Alimentari. Grugliasco (TO) 18th May 2017. Supervisors: Dr Marco Porporato and Dr Daniela Laurino.
- **Bee-institute Kirchhain** (DE). From June 25th to July 07 2017. Supervisors: Dr Ralph Büchler and Marina Meixner.
- **CREA-API.** Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria – unitá di ricerca in apicoltura e bachicoltura. Reggio-Emilia (RE) Italy. 22-23 November 2016 and 5-7 December 2016. Supervisor: Dr Cecilia Costa.

Assistant supervisor of following thesis:

- **"Aspetti del comportamento delle api: meccanismi di difesa verso I patogeni e verso l'ambiente"**. Corso di Laurea in Allevamento e Benessere Animale. A.Y. 2014-2015 Student: Chiara Coatti. Supervisor: Prof. Rita Rizzi
- **"L'allevamento dell'ape regina"**. Corso di Laurea in Produzioni Animali, Alimenti e Salute. A.Y. 2015-2016. Student: Laura Colombo. Supervisor: Prof. Rita Rizzi.
- **"L'utilizzo delle api nel rilevamento di esplosivi"** Corso di Laurea in Allevamento e Benessere Animale. A.Y. 2015-2016 Student: Elena Domina. Supervisor: Prof. Rita Rizzi
- **"Effetto dell'andamento climatico sulle caratteristiche produttive e comportamentali di api alleviate in provincia di Lodi"** Corso di Laurea Magistrale in Scienze e Tecnologie delle Produzioni Animali. A.Y. 2016-2017. Student: Chiara Mattioli. Supervisor: Prof. Rita Rizzi
- **"Qualità Morfometrica e Riproduttiva in Regine di *Apis mellifera*"** Corso di Laurea Magistrale in Scienze e Tecnologie delle Produzioni Animali. A.Y. 2016-2017 Student: Eleonora Bergonzoli. Supervisor Prof. Giulio Pagnacco.
- **"Studio della variabilità dello stadio pupale di *Apis mellifera*"** Corso di Laurea in Allevamento e Benessere Animale. A.Y. 2017-2018. Studen: Laura Nalon. Supervisor: Prof. Michele Mortarino.
- **"Studio sulla variabilità fenotipica e genetica di api regine: caratteri morfologici e riproduttivi"** Corso di Laurea Magistrale in Scienze e Tecnologie delle Produzioni Animali. A.Y. 2016-2017. Student: Maria Grazia Deiorio. Supervisor: Prof. Giulio Pagnacco.

Other relevant activities:

- "Hygienic behavior in honey bee: from phenotype to genomics**" Invited lecturer** from Prof. Maria Lina Longeri for unit "Genomic analysis of inherited diseases" - Master in Veterinary Biotechnology Science – University of Milan on May 18, 2018 (two hours).
- "Hygienic behavior in honey bee: from phenotype to genomics**" Invited lecturer** from Prof. Maria Lina Longeri for unit "Genomic analysis of inherited diseases" - Master in Veterinary Biotechnology Science – University of Milan on May 25, 2017 (two hours).
- **Tutoring activity** during course "Matematica e Fisica" unit "Matematica e Statistica" held by Prof. Rita Rizzi. From 11 October 2016 to 21 December 2016. (eight hours).
- "Un progetto di miglioramento genetico" **Invited lecturer** during "Convegno e Festa dell'Apicoltore" organized by FAI – Federazione Apicoltori Italiani and APAT-Apicoltori in Veneto. Treviso, 4th December 2016.

Publications (abstracts, conferences contributions and full paper)

- **Elena Facchini**, Laura Nalon, Maria Elena Andreis, Mauro Di Giancamillo, Rita Rizzi, Michele Mortarino. 2018. Honey bee pupal length assessed by CT-scan technique: effects of *Varroa destructor* infestation, developmental stage and spatial position within the brood comb. Submitted to *Scientific Reports*
- **Elena Facchini**, Piter Bijma, Giulio Pagnacco, Rita Rizzi, Evert Willem Brascamp. 2018. Hygienic behaviour in honey bees: a comparison of two recording methods and estimation of genetic parameters. Accepted for publication in *Apidology*
- **Elena Facchini**, Marco Zetti, Livio Colombari, Maria Elena Andreis, Mauro Di Giancamillo, Rita Rizzi, Michele Mortarino. 2018. Exploring tolerability and efficacy of formic acid for varroa control: standard and alternative approaches. XXX SOIPA Congress Milano, Italy. 26-29 June 2018
- **Elena Facchini**, Maria Elena Andreis, Rita Rizzi, Mauro Di Giancamillo. 2017. Catch me if you s-can! Computed tomography analysis on a brood comb. 13th Coloss Conference, Athens, Greece. 2-3 November 2017.
- **Elena Facchini**, Rita Rizzi, Giulio Pagnacco, Giulietta Minozzi. 2017. Estimation of genetic parameters for honey production in the honey bee – Preliminary results. Italian Journal of Animal Science, 16, suppl. 1, p.118-119.
- **Elena Facchini**, Michele Mortarino, Francesca Dell'Orco, Rita Rizzi. 2017. Expression monitoring of relevant sensitivity genes in honey bee antennae and their relationship with Hygienic Behavior. Proceeding of Veterinary and Animal Science Days 2017, 6th- 8th June, Milan, Italy.
- Francesca Dell'Orco, Francesca Albonico, Monica Loiacono, **Elena Facchini**, Rita Rizzi, Michele Mortarino. 2016. Expression analysis of relevant gene targets for Hygienic Behaviour in honey bees (*Apis mellifera*). SOIPA Bari 2016.
- Michele Mortarino, Mattia Blonda, Sergio Zanzani, Francesca Dell'orco, **Elena Facchini**, Rita Rizzi. 2016. Effect of Neem (*Azadirachta indica*) oil on varroa mite development in field conditions. The 7th European Conference of Apidology Cluj-Napoca 7-9 September 2016. Ed. Daniel S. Dezmirean.
- **Elena Facchini**, Emanuela Arrigoni, Flavia Pizzi, Francesca dell'Orco, Michele Mortarino, Giulietta Minozzi, Giulio Pagnacco, Rita Rizzi. 2016. Hygienic Behaviour in honey bee: a comparison of two assays. The 7th European Conference of Apidology Cluj-Napoca 7-9 September 2016. Ed. Daniel S. Dezmirean.

- Sara Panseri, Federica Borgonovo, Marcella Guarino, Lucia Piana, **Elena Facchini**, Michele Mortarino, Luca Chiesa, Rita Rizzi. 2016. Effect of the storage temperature on volatile organic compounds and aroma profile of *Robinia pseudoacacia* honey. The 7th European Conference of Apidology Cluj-Napoca 7-9 September 2016. Ed. Daniel S. Dezmirean.
- Francesca Dell'Orco, Monica Loiacono, Francesca Albonico, Sergio Zanzani, Antonella Cersini, Giovanni Formato, **Elena Facchini**, Mario Colombo, Michele Mortarino. 2016. Real time PCR coupled to High Resolution Melting Analysis for detection and quantitation of *Nosema ceranae* in honey bees. The 7th European Conference of Apidology Cluj-Napoca 7-9 September 2016. Ed. Daniel S. Dezmirean.
- **Elena Facchini**, Michele Mortarino, Francesca Dell'Orco, Rita Rizzi. 2016. Hygienic Behaviour in honey bee: a comparison of two in-field assays for phenotypic characterization. Proceeding of Veterinary and Animal Science Days 2016, 8th- 10th June, Milan, Italy.
- Francesca Dell'Orco, **Elena Facchini**, Giovanni Cilia, Rita Rizzi, Michele Mortarino. 2016. Candidate molecular markers of hygienic behaviour in honey bees (*Apis mellifera*): an expression study. Proceeding of Veterinary and Animal Science Days 2016, 8th- 10th June, Milan, Italy.
- Michele Mortarino, Livio Colombari, Giovanni Prestini, **Elena Facchini**, Giovanni Formato. 2016. Results of a WG4-FA trial in Northern Italy. Varroa control taskforce-workshop-assessment of alternative methods for varroa control- Unije, Croatia, 19th – 20th May 2016.