EFFECTS OF LARVAL AGE AT GRAFTING AND SUPPLEMENTAL FEEDING ON MORPHOMETRICS AND OVIPOSITION IN HONEYBEE QUEEN (*Apis mellifera scutellata*, *Hymenoptera: Apidae*) IN KARURA FOREST, KENYA

LOISE KAWIRA NJERU

MASTER OF SCIENCE

(Zoology)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2018

Effects of Larval Age at Grafting and Supplemental Feeding on Morphometrics and Oviposition in Honeybee Queen (*Apis mellifera scutellata, Hymenoptera: Apidae*) in Karura Forest, Kenya

Loise Kawira Njeru

A Thesis Submitted in Partial Fulfillment for the Degree of Master of Science in Zoology in the Jomo Kenyatta University of Agriculture and Technology

2018

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature: ……………………………………… Date: ………………………

Loise Kawira Njeru

This thesis has been presented for examination with our approval as the appointed University supervisors.

Signature: ……………………………………… Date: ………………………

Prof. Helen L. Kutima, PhD

JKUAT, Kenya

Signature: ……………………………………… Date………………………

Prof. Suresh K. Raina, PhD

ICIPE, Kenya

DEDICATION

This work is dedicated to the lord God Almighty, my family and friends for the support they gave me throughout the study.

ACKNOWLEDGEMENTS

I highly appreciate Prof. Helen Lydia Kutima for accepting to be my university supervisor and for her continuous guidance and great input towards the production of a high-quality research. She always motivated me throughout the study period and I will forever remember her for her patience and encouragement throughout the study.

I am greatly indebted to Prof. Suresh Kumar Raina my icipe supervisor and the Team Leader of the Bee Health Project of the International Center of Insect Physiology and Ecology (ICIPE) for introducing me to the intriguing world of bees, and for his technical guidance, critical comments, and moral support throughout the research period. I would like to thank the Jomo Kenyatta University of Agriculture and Technology (JKUAT) and especially the Department of Zoology for accepting my registration as a Postgraduate student and for the assistance provided throughout my study period.

I am highly thankful to the International Center of Insect Physiology and Ecology (icipe), the Director General Dr. Segenet Kelemu for providing all the research facilities and a favourable environment that I enjoyed throughout the study. I thank the European Union (EU) under grant number EU/ICIPE 2013/313-659 and the International Fund for Agricultural Development (IFAD) under grant number IFAD/ICIPE 1229 for the financial support which enabled me to achieve this study. I am grateful to Dr. Daisy Salifu of ICIPE Biostatistics unit for her scientific guidance. I thank Dr. David Cham of ICIPE for his outstanding contribution to this work. Many thanks to the Bee Health

technicians: James Ng'ang'a, Joseph Kilonzo, Albert Baya and Newton Ngui for their invaluable assistance during data collection.

I would also like to express my gratitude to the entire management of Kenya Forest Services (KFS), for allowing me to establish the experimental apiaries at Karura Forest. I also express my heartfelt appreciation to my entire family not forgetting my son Emmanuel and daughter Marvel for their love and understanding which encouraged me throughout the study, my sincere thanks goes to my caring husband David Mugendi for his incalculable support in all aspects throughout the study.

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ABBREVIATIONS AND ACRONYMS

A I	Artificial Insemination
ANOVA	Analysis of Variance
CRD	Completely Randomized Design
CPF	Colony Performance Factor
EU	European Union
FKB	Freezer-Killed Brood
GLM	Generalized Linear Model
GPS	Geographic Positioning System
ICIPE	International Centre of Insect Physiology and Ecology
IFAD	International Fund for Agricultural Development
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KFS	Kenya Forest Services
LNKB	Liquid Nitrogen-Killed Brood
MANOVA	Multivariate Analysis of Variance

Masl Meters above sea level

N M Natural Mating

QMP Queen Mandibular Pheromone

SNK Student- Newman- Keuls

SV Spermatheca Volume

µl Microlitre

χ^2 Chi–square

DEFINITION OF TERMS

Artificial insemination:	Collection of drone semen and introducing it into the queen's genital tract using a capillary
Colony Performance Factor:	This is the total score of the selection parameters scored during the colony evaluation rating.
Impulse:	The driving force.
Larval Grafting:	The transfer of young larva which is less than 24 hours old from the natural worker cell to artificially made cell cups.
Morphometrics:	Measurements of the form of an organism.
Natural mating:	The process of the honeybee queen flying to the drone congregation area.
Nuptial flight:	The actual flight when the queen bee goes out for mating.
Oviposition:	Egg laying.
Pheromone:	A chemical substance used by bees for communication.

Retinue behaviour:	The act of surrounding the queen by the worker bees in response to the pheromone being produced by the queenbee.
Supplemental feeding:	Feeding of experimental colonies with additional pollen diet.
λ:	Wilks' Lambda test.

ABSTRACT

In Africa, honeybees provide critical pollination services, nutrition and income for smallholder farmers. However, because of pests and pathogens honeybees are under threat of population decline. Moreover, lack of adequate research on the existing queen rearing technologies has led to decline in honeybee queen quality. The honeybee queen is the repository of a colony's heritable genetic traits, its superiority determines the productivity and resilience of the colony. Effects of factors such as age of grafted larva, supplemental feeding and mating are not known for African bee races. To bridge this gap, a study was undertaken at the International Centre of Insects Physiology and Ecology (ICIPE) Karura forest apiaries in Kenya to determine the effect of larval age and supplemental feeding on morphometrics and oviposition in the honeybee queen *Apis mellifera scutellata*. Queens were reared in 12 colonies with two feeding regimes. Five larval age groups, 6, 12, 24, 36, and 48 hours old were used to raise queens. Specific morphometric parameters of the queens were determined. The wet weight, spermatheca volume and the external parameters (head length and width, wing length and width and thorax length and width) of the emerged queens were recorded. Oviposition rate in Naturally Mated queens (NM) and Artificially Inseminated queens (AI) was determined by recording the number of eggs laid daily. Multivariate Analysis of Variance (MANOVA), Analysis of Variance (ANOVA), and Student-Newman-Keuls (SNK) test were used to evaluate the effect of larval age and supplemental feeding on queen quality. The oviposition rate of naturally mated and artificially inseminated queens was evaluated using t- test. Results showed that age of grafted larvae and supplemental feeding significantly affect the morphometrics of the reared queens ($p = 0.001$). The 24-hour-old larvae were heavier and larger in most of the external parameters and thus it is the optimal age for grafting in *A. m. scutellata* queens. Oviposition rate was the same in both the NM and AI queens. Further study is required to establish any correlations between the quality of the queens produced from the different age groups and overall colony productivity.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Bees have been found to be more speciose and abundant in warm-temperate xeric parts of the world (Michener, 2000). There are three races of the honeybee *Apis mellifera Linnaeus* in Kenya (*A. m. scutellata, A. m. monticola and A. m. litorea*) which differ from each other with respect to size, cubital index and abdominal colour banding pattern (Raina & kimbu, 2007).

Honeybees are important pollinators and are responsible for much of the world's agricultural production and the conservation of biodiversity. Pollinators are essential contributors to global nutrition and food security. An estimated three quarters of major global food crops benefit from pollinators (Klein *et al*., (2007), and about one third of all plants or plant products eaten by humans depend directly or indirectly on bees for their pollination (Bradbear, 2009). Pollination is one of the first and most important steps in fruit production and for almost 90% of angiosperms (Ollerton *et al*., 2011). This vital ecological service is facilitated through insect vectors mainly bees (Kevan and Baker, 1983; Michener, 2007). The honeybee species *A. mellifera* is considered one of the most important generalist pollinators of both agricultural and natural ecosystems. In 2005 worldwide estimates of the total economic value of pollination by honeybees stood at €154 billion (Moritz *et al*., (2010).

Global pollinator declines have been attributed to habitat destruction, pesticide use, pests/parasites and pathogens and climate change (Neumann & Carreck, 2010) or some combination of these factors, and managed honeybees, *Apis mellifera*, are part of worldwide pollinator declines thus endangering pollination (Aizen & Harder, 2009; Aizen *et al,* 2009; Meixner, 2010; Pettis *et al*, 2012; Zee *et al*., 2014).

The three castes (queen, worker and drone) in a honeybee colony are a single queen, a few hundred drones and several thousand workers. Among the members of the colony, there is a division of labor and specialization in the performance of biological functions. The/ (Winston, 1992) and collectively they make up the honeybee colony (Zawislak & Burns, 2013). Workers can flexibly shift among different tasks, depending upon colony need (Fergusson and Winston, 1988; Smith *et al*, 2008).

The queen honeybee is central to a colony's survival and function. She is the only fertile female in the colony capable of laying eggs that will hatch into offspring to keep the colony thriving. The worker bee is infertile, and if forced to become a laying worker bee (e.g. in the absence of a queen), is capable of only producing male (drone) brood from unfertilized eggs (due to the haplodiploid sex-determination system found in honeybees (Breed, 2003; Collins & Evans, 2006).

The chemical pheromones produced by a queen bee impart a unique identity to each colony and its members. The presence of these pheromones also keeps the colony cohesive and orderly. Chemical communication is an important area of physiology of the

honeybee (*A. mellifera*) and the highly complex social organization of honeybees is mediated through pheromones. The Queen Mandibular Pheromones (QMP) signals presence of the queen to workers either by direct contact with the pheromone source (the queen) or indirect serial transmission of QMP from worker to worker displaying a behaviour called retinue behaviour (Keeling *et al*, 2003; Trhlin & Rajchard, 2011; Zawislak & Burns, 2013).

A queen bee is larger than a worker bee, having a large pea-sized thorax and long tapered abdomen (Johnstone, 2008). There is usually only one mature queen per colony at a time and she is responsible for reproduction in the colony. A mature queen may lay 1500 to 2000 eggs a day (Gencer *et al.,* 2000) but averages 700–1000 a day when conditions are unfavorable. A queen can live for several years, although under commercial conditions she is usually replaced by beekeepers every 1 or 2 seasons. Young queen has more vigor, which translates to better colony health. After emerging from her cell, the virgin queen mates on the wing six to ten days later with up to 20 drones (Johnstone, 2008). The sperms are then stored in a sperm sac called spermatheca, and the sperm are used to fertilize the eggs throughout her lifetime (Breed, 2003; Collins & Evans, 2006).

A queen is the source of a colony's heritable genetic traits. These traits influence many aspects of colony behaviour, such as their defensiveness, parasite tolerance, disease resistance, productivity and the rate of population growth. The queen honeybee is fundamental to a colony's survival and function (Zawislak & Burns, 2013). Tarpy *et al,* (2000) reported that the reproductive quality of queens reared from younger larva are

higher. A high-quality queen should have heavy body weight, large number of ovarioles, a large spermatheca (Carreck *et al*., 2013, Human *et al*., 2013) and be free from diseases and pests (Hatjina *et al*., 2014).

Beekeepers tend to raise queens from older larvae since they are easily visible. According to (Mahbobi *et al*., 2012) queen bees reared from 1-day-old larvae were significantly heavier at emergence (158.83 mg) and had significantly larger spermatheca (0.99 mm3) than queens reared from 2 and 3day old larvae. Queens reared from 1-day-old larvae were of the highest quality and the age of the larva significantly affected the morphological characteristics of reared queens, and thus, their quality. Gilley *et al.* (2003) produced variation among the quality of related morphological traits by rearing queens from worker larva of different ages. Many of these traits correlated with each other.

Honeybee regimens and diet formulations are a common approach in beekeeping where bees are artificially fed with syrup, pollen or vitamin supplements (Gençer *et al*., 2000), or carbohydrates and proteins (Hussein *et al.,* 2000). Therefore, supplemental feeding affects queen quality. Król *et al.* (1992) concluded that queens reared in colonies that were fed with sugar syrup supplemented with vitamin B, were 11% heavier and had 6% more ovarioles. In queen rearing, pollen, nectar and syrup are important nutritional factors. Lack of pollen reduces the number of drones reared (Estegamat & Gholami, 2010). Mahbobi *et al.* (2012), reported that supplemental feeding significantly increased most morphological characteristics of the reared queens. Supplemental feeding and larval

ages did not affect the wing length of *A.m. meda.* It is commonly believed that artificially inseminated honeybee queens initiate oviposition much later than naturally mated queens (Woyke *et al.,* 2008). However, numerous studies have demonstrated that artificially inseminated queen bees have equal performance compared to naturally mated queen bees (Cobey, 2007). In view of the above stated information this study was undertaken to determine the optimal age to rear healthy queens and productive colonies to establish African breeding lines for *A. m. scutellata* in Karura forest.

1.2 Statement of the problem

Efforts to improve beekeeping in Kenya by various stakeholders are usually based on modern equipment and training of beekeepers on apiary maintenance and colony management without addressing colony performance improvement which is due to lack of adequate research on the beekeeping technologies in Kenya. To improve colony productivity, there is need to focus on the bees, especially the honeybee quality i.e. keeping bees with specific traits. There is lack of adequate and intense research on the existing beekeeping technologies in Kenya. With the growing pressure on the environment due to climate change, pesticide use, habitat loss and fragmentation and associated loss of honeybees, these pollinators are under threat of population decline in Kenya.

Lack of experience in queen rearing and colony management by Kenyan beekeepers means that beekeepers cannot dequeen and requeen colonies as one way of managing

colonies for honey production hence, the potential for selective breeding has not been explored. Therefore, there exists a need for proper queen rearing techniques for colony improvement which will form the foundation for selective breeding.

1.3 Justification of the study

Bees are important pollinators and many ecosystems depend on pollination by bees for their existence and for increasing their genetic diversity.

A decline in bee colonies and bee species could, therefore, threaten the survival of plant species that depend on pollination by bees since some types of plants depend entirely on bee pollination.

Queen rearing is not commonly practised in Africa and especially in East Africa by the beekeepers. This is due to lack of knowledge and skills on queen rearing and colony multiplication through colony division. This therefore limits the beekeepers on essential practices of colony manipulation such as dequeening and requeening which can be used to improve the colony performance (Forster, 1972).

Knowledge on queen rearing will serve to; improve colony productivity in terms of effective pollination and income from hive products such as honey, royal jelly, pollen, wax, propolis, bee venom and package bees and in conservation strategies.

This study addresses the issues by determining variability in some selection parameters within the general population during breeder colonies selection. It will assess various

factors that may affect queen quality such as age of grafted larvae, origin of the larvae, presence or absence of food in the starter and finisher colonies and mating.

Little is known on the effect of the above factors on the quality of the African honeybee queens, hence the need for carrying out the study. A well-explored and documented investigation on the above-mentioned factors will open the way for queen bee breeding in Africa.

1.4 Hypotheses

1. Larval age at grafting has no effect on the morphometrics of the resultant honeybee queens of *A. m. scutellata* in Karura Forest.
2. Supplemental feeding of nurse colonies has no effect on the morphometrics of the resultant honeybee queens of *A. m. scutellata* in Karura Forest.
3. The oviposition behaviour of the artificially inseminated queens is not different from that of naturally mated queens in *A. m. scutellata* in Karura Forest.

1.5 General objective

To assess the effect of larval age and supplemental feeding on morphometrics and oviposition behaviour of naturally mated and artificially inseminated queens of *A.m. scutellata* in Karura Forest, Kenya.

1.6 Specific Objectives

1. To assess the effects of larval age on the morphometrics of the honeybee queens of *A.m. scutellata* in Karura Forest.
2. To determine the effects of supplemental feeding on morphometrics of the honeybee queens of *A.m. scutellata* in Karura Forest.
3. To evaluate the oviposition behaviour of naturally mated and artificially inseminated queens of *A.m. scutellata* in Karura Forest.

1.7 Research questions

1. Does the larval age affect the morphometrics of the honeybee queen of *A. m. scutellata*?
2. Does supplemental feeding affect the morphometrics of the honeybee queen of *A. m. scutellata*?
3. Is there any variation in oviposition behaviour of artificially inseminated and naturally mated queens of *A. m. scutellata*?

1.8 Scope of the study

This study focused on adopting and providing information pertaining to queen rearing techniques in African honeybee sub- species. *Apis mellifera scutellata* was used as the model since it is the most widely distributed honeybee sub-species in Africa. The study involved raising queens from different larval age groups to determine the larval age

which give rise to high quality queens. Feeding regime was also tested to determine if it contributed to the queen quality. Comparison of the egg laying performance of artificially inseminated and naturally mated queens was also carried out.

This research was conducted during the months of November 2014 to May 2015 at the ICIPE apiaries 1 and 2 situated in Karura forest Nairobi Kenya. Variability in parameters for breeder colony selection criteria were also evaluated in thirty- four African honeybee colonies of *A.m. scutellata* according to Gregorc and Lokar (2010).

CHAPTER TWO

LITERATURE REVIEW

2.1 General introduction

There are more than 11 extant species within the genus *Apis* worldwide (Michener, 2000) that are classified into two groups, based upon nesting structures and activities. The first group builds single comb, open-air nests, i.e. *A. andreniformis*, *A. florea, A. dorsata, A. breviligula*, *A. binghami* and *A. laboriosa.* These bees are restricted to the Asian tropics and subtropics. The second group consists of species that nest inside cavities where they build multiple combs. These are *A. cerana, A. koschevnikovi, A. nigrocincta, A. nuluensis,* and *A. mellifera* (Hepburn and Michener, 2000; Radloff, 2011). There are 32 sub-species of *Apis mellifera* worldwide out of which 14 are African (Engel, 1999; Amssalu *et al.*, 2004).

African honeybee races are infested with *Varroa* mites (Begna, 2015) but some show resilience against them (Muli *et al.*, 2014). Though resilient to the *Varroa* mite, native bee populations have also been declining, likely due to the combined influences of the pests and diseases, habitat fragmentation, urbanisation and pesticides (Aizen & & Feinsinger, 1994; Cane, 2001; Roubik, 2001).

In the natural mating process, honeybee queens mate in flight with numerous drones from diverse genetic resources, but it is impossible to identify the colonies from which

drones that mated with the queen came from (Jensen, 2000; Cobey, 2007). The ability to control mating has been one of the most challenging aspects of honeybee breeding (Cobey, 2007). By selectively raising their own queens, beekeepers can take control of the characteristics they desire in their own bee stocks (Hayes, 1991; Zawislak, & Burns, 2013). Beekeepers have developed techniques to rear large numbers of queen bees to requeen colonies regularly (every year or two years), to reduce swarming, to increase brood and honey production, to start new colonies, and to change certain genetic characteristics (Ruttner, 1983; Laidlaw & Page, 1997). Many economically important traits of the honeybee have medium to high heritabilities and are therefore capable of strong response to selection. However, the natural mating system of honeybees makes it difficult to exclude unselected males from mating and necessitates expensive procedures like artificial insemination or isolated mating stations (Oxley *et al*., 2010). The number of drones ready to compete for mating with virgin queens in a drone congregation area ranges from 10,000 to 15,000 (Koeniger *et al.,* 2005), coming from about 240 colonies in surrounding apiaries (Baudry *et al*., 1998). Controlled mating is the foundation of all stock improvement programmes. This has been difficult to control in honeybees because they multiple mate in flight. Artificial insemination has solved this problem, providing an essential tool (Cobey, 2007). This also enables a degree of selection for desirable colony characteristics (Cobey, 2005).

2.2 Scientific classification of Honeybee

The honeybee belongs to the Domain: Eukaryota, Kingdom: Animalia, Phylum: Arthropoda

Subphylum:Uniramia, Epiclass:Hexapoda,Class:Insecta,Order: Hymenoptera Suborder: Apocrita, Superfamily: Apoidea, Family: Apidae , Subfamily:Apinae, Tribe:Apini, genus:*Apis*

Subgenus:*Apis*, Species: *A. mellifera*. There are many subspecies of *Apis mellifera* native to Europe, Africa and the Middle East. The trinomial name for the subspecies model used in this study was *Apis mellifera scutellata* (Lepeletier, 1836; Ruttner, 1988).

2.3 Origins and Historical Perspective of honeybees

The African honeybee (*Apis mellifera scutellata*) is a subspecies (race) of the western honeybee. It is native to central and southern Africa (Ruttner, 1988). The appearance of the African honeybee is very similar to the European honeybee. However, the African honeybee is slightly smaller (Kaplan, 2004; Crane, 2013). The average body length of a worker is 19 mm. Its upper body is covered in fuzz, and its abdomen is ringed with black stripes (Kaplan, 2004). African bees defend their hive faster than the European honeybees and they swarm more often than the European honeybees and were imported to Brazil in 1950s for experiment and some escaped and started colonies. Can chase you for over a quarter of a mile.

2.4 Sex determination in honeybees

The honeybee (*Apis mellifera*) employs an interesting system in which sex is determined (Gempe *et al*., 2009). Haplodiploidy (arrhenotoky) is a sex-determination system in which males develop from unfertilized eggs and are haploid, and females develop from fertilized eggs and are diploid (Beukeboom *et al.,* 1995). In honeybees, the drones (males) are entirely derived from the queen, their mother. The diploid queen has 32 chromosomes and the haploid drones have 16 chromosomes. The honeybee (*Apis mellifera*) forms two female castes, the queen and the worker. This dimorphism depends not on genetic differences, but on ingestion of royal jelly. Royalactin a protein in royal jelly, induces the differentiation of honeybee larvae into queens by increasing the body size and ovary development and shortening developmental time in honeybees (Kamakura, 2011).

In the eusocial honey bee *Apis mellifera*, with reproductive queens and sterile workers, a female larva's developmental fate largely depends on the diet it receives. Larvae fed exclusively royal jelly, a glandular secretion of nurse bees, become queens, whereas those fed royal jelly for 3 days and subsequently honey and beebread become sterile workers (Mao *et al*., 2015)

2.5 Life cycle of the honeybee

Honeybees develop through complete metamorphosis from egg to larva to pupa and adult. This development occurs in the cells of the wax comb (Li *et al*., 2007). The queen

develops from a fertilized egg. A queen is reared in a special cell that hangs vertically and extends as the larva grows. Throughout the queen development and life, it is fed with a diet of royal jelly. The queen cell is capped on day 5, then the larva spins cocoon. The development is completed in 16 days after the egg is laid (Ellis & Ellis, 2009). The worker develops from a fertilized egg and it is not fully developed sexually. The worker and the drone larva are fed with royal jelly up to 3 days old then fed with bee bread (honey and pollen) the rest of their lives. The worker development is completed in 21 days after the egg is laid. The drone development is completed in 24 days after the egg is laid (Yadav *et al*., 2017).

2.6 Economic importance of honeybees

Beekeeping is a relatively inexpensive activity that generates additional or complementary income for rural households and contributes to the overall household food and income security (Bradbear, 2009). Compared to other agriculture land-based enterprises, beekeeping requires very little, land and labor. In addition to honey, there is a range of useful and marketable bee produce, such as wax, propolis, pollen, royal jelly and bee venom (Raina, 2004; Muli *et al*., 2005; Raina *et al.,* 2009).

Humans have relied on bees since time immemorial to provide pollination services to the crops. Pollination is an ecosystem service that economically has both ecological and agricultural values. Ecological values are portrayed in the regulatory functions provided by an ecosystem, e.g. supporting the reproduction success of different plants. Plants

support many life forms that benefit human beings through their use and non-use values (Kevan & Phillips, 2001).

The benefits from pollination are most notably important to crop production Klein *et al.,* (2003), Kasina (2007), and Kiatoko *et al.* (2014) successfully correlated increased yields of fruit with increased diversity and abundance of pollinators. Pollination, for the productivity and health of agricultural crops, is of great importance (Kasina, 2007). Man has used honey since ancient times as a source of food, medicine and for religious and cultural ceremonies. In Kenya stingless bee honey is popular due to its medicinal properties (Macharia, 2008; Raina *et al*., 2011).

2.7 Challenges facing honeybees

Honeybee colonies are declining globally thus endangering pollination and other services (Zee *et al*., 2014). There are three major drivers of colony losses, and these are; environmental stressors which involves factors such as climate change, pesticide use and habitat destruction. Genetic diversity and vitality and pests/parasites and pathogens. These factors may lead to colony loss individually or as an interaction of factors within one major driver or an interaction between all the factors (Neumann & Carreck, 2010). *Varroa* mite is one of the major causes for selective pressure in bees globally, although African bees have been reported to be more resilience to v*arroa* mite than European bees (Muli *et al.,* 2014), it is therefore important to take precautions for improvement and conservation strategies through queen breeding (Cobey, 2005).

The honeybee is the key pollinating agent for approximately 52 of the leading 115 global food commodities (Klein *et al*., 2007). Honeybees are, therefore, undoubtedly the most important managed pollinator (Morse, 1991). Recently drastic declines of managed honeybee populations have been recorded (Allen-Wardell *et al*., 1998; Potts *et al.,* 2010) and consequently concerns regarding the sustained pollination of agricultural food crops persists worldwide (Allen-Wardel *et al*., 1998; Oldroyd, 2007; Neumann & Carreck, 2010).

2.8 Queen rearing

Queen rearing is the process of producing virgin queens in a honey bee colony that uses an existing queenless (without a queen) or a queenright (with a queen) colony. For successful managing and rearing of queen bees it is imperative to adapt beekeeping measures to colony development (Wei *et al.*, 2003; Cobey 2007). The first queen rearing was practiced in ancient Greece, where beekeepers put combs with young larvae into queenless colonies to raise emergency queen cells (Büchler *et al*., 2013). Techniques of rearing queens have been developed to allow the beekeepers to produce good stock and to replace old and undesirable queens in their colonies (Ruttner, 1988; Cobey, 2007). A well-mated and well-fed queen can lay about 2000 eggs/day during the flowering period. A queen lays a fertilised or unfertilised egg according to the width of the cell (Mattila & Seeley, 2007).

The young queen larva develops differently because it is more heavily fed with royal jelly, a protein rich secretion from glands on the young workers. If not heavily fed the larva becomes a regular worker bee (Jensen, 2000).

2.8.1 Natural queen rearing impulses

Bee colonies raise queens naturally. Inducing a colony to rear queens merely encourages this natural phenomenon, subject to the beekeeper's conditions and schedule. There are three natural conditions in the hive under which bees rear their own queens. These are known as the supersedure, swarming and emergency impulses, (Zawislak & Burns, 2013).

2.8.1.1 Supersedure impulse

The bees perceive the queen to be failing. When a queen is beginning to fail from old age or some other infirmity, the bees seem to realize that she cannot be with them much longer, so they take steps toward rearing for themselves a new mother. Queen-cells are started. They give the larvae plenty of food, but usually do not build more than three or four cells. Bees select a larva and begin feeding it with royal jelly. They build a supersedure cell around it, which hangs down from the face of the comb (Smith, 1923; Zawislak & Burns, 2013).

2.8.1.2 Swarming Impulse

The natural way of reproduction in which the bees multiply their colony numbers. When a colony is preparing to swarm it starts many queen-cells in which the queen lays eggs. These cells are formed along the edges of the brood combs, often overhanging the bottom bar of the frames, usually 10–15 swarm cells. When the first cell is capped, if the weather is favorable, the swarm usually comes out. As swarming occurs when the colony is at its height of brood rearing, the larvae are well supplied with royal jelly, so that the finest queens are reared. They not only build large numbers of cells but also supply the larva in them lavishly with food. During swarming season, the old queen leaves with the prime swarm before the first virgin queen emerges from a queen cell (Ahmad *et al*., 2013).

2.8.1.3 Emergency impulse

There is suddenly no queen in the honeybee colony. The bees build many cells, but they do not give them the proper attention and ration the food for the larva. Emergency cells may be anywhere in the brood nest, although a group of two to three cells in a central position on the comb is common (Smith, 1923; Zawislak & Burns, 2013).

2.8.2 Queen rearing techniques

Queen rearing is a process of raising honeybee queens that uses an existing queenless or a queenright colony. It encourages the reproduction of queens with characteristics that

help bees to thrive in specific climatic and geographic conditions (Ruttner, 1988; Morse, 1994). Several methods exist which are applied in rearing queens:

2.8.2.1 Miller method

Miller method involves trimming of the comb which is placed at the middle of a brood box of a selected colony. The queen lays in it and the bees extend the bottom and fill the gaps, allowing the queen to lay in the extensions a few days later. When the eggs start to hatch the comb is removed from the colony, the bees removed, and the comb is cut back to where the larvae are 24 – 36 hours old that is 4 - 4 1/2 days from the egg being laid. The comb is then placed in a cell raising colony and the bees build queen cells on the exposed edge as quoted by (Johansson & Johansson, 1973).

2.8.2.2 Alley method

A strip of the comb is made to lay on a flat board and cut almost to the midrib on the opposite side to where you want the queen cells to be built with a sharp knife. The comb (or several pieces) are fixed to a strip of wood that is nailed to a frame. Queen cells are then built at random and often joined, destroy 2 -3 larvae between the selected ones before placing the frame in the cell raising colony. When the queen cells are ready to be distributed they are cut out as described by (Laidlaw & Eckert, 1962).

2.8.2.3 Doolittle method

This involves grafting of the young larvae from the worker cells into artificial queen cell cups as quoted by (Büchler *et al*., 2013). It is preferred because it is quick, cheap and reliable. It ca be applied in mass production of queens and therefore employed by commercial beekeepers worldwide to date. This is the method which was adopted in this study.

2.8.3 Economic importance of queen rearing

Healthy queen bees help to reproduce colonies that carry desirable traits such as resistance to pests and diseases, high levels of honey productivity and effective pollination capabilities.

Queen rearing replaces queens that are failing or have died, increases colony numbers through colony division and helps to re-queens hives that have become aggressive (Johnstone, 2008).

Rearing queens is one of the most rewarding aspects of beekeeping.

It provides a means to maintain young, vigorous queens in colonies and is the foundation of good colony management. This also enables a degree of selection for desirable colony characteristics (Cobey, 2007; Gregorc & Lokar, 2010).

2.9 Hygienic behaviour of honeybees

Hygienic behaviour is the uncapping of brood cells containing dead or diseased brood and the subsequent removal of the remains of this brood (Spivak & Reuter, 1998; Fries & Lindström, 2010; Nicodemo *et al*., 2013).

Hygienic behaviour is a genetic trait of honeybees. It is the main defence against American foulbrood and American chalkbrood and is one defence against *Varroa Mites*. Testing for this trait is simple. It involves freezing a section of sealed pupae and recording how many dead pupae the bees remove within 24 hours (Spivak & Reuter, 2001). Resisting disease is an economically important form of social immunity in a honeybee colony (Wilson-Rich *et al.,* 2009).

According to Spivak and Reuter (2001), testing for hygienic behaviour can be performed in two ways Freezer-Killed Brood test (FKB) and the Liquid Nitrogen-Killed Brood test (LNKB). With either method, use 3 to 10-day-old pupae (just pupating to light tan colour). The best tool for breeding for disease tolerance in honeybees is presently to select for breeder queens exhibiting a pronounced level of hygienic behaviour. This leads to increased resistance to all known brood diseases and to decreased *Varroa* population build up (Fries & Lindström, 2010).

To evaluate Hygienic behaviour, a portion of the sealed brood comb will be frozen with liquid nitrogen and returned to the hive for cleaning. The rate of cleaning will be checked after 24 hours, Colonies that are considered hygienic based on the FKB assay, i.e.

colonies that remove >95% of the FKB within 24 hours, will show high consistency in results between assays, irrespective of strength of colony and nectar flow (Büchler *et al*., 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.2 Study site

The study was conducted at Karura forest in Kenya at ICIPE apiary 1, GPS coordinates 36.83470E - 1.234420S and ICIPE Apiary 2 GPS coordinates 36.814160E- 1.245250S Elevation 1715 m (Figure 3.1).

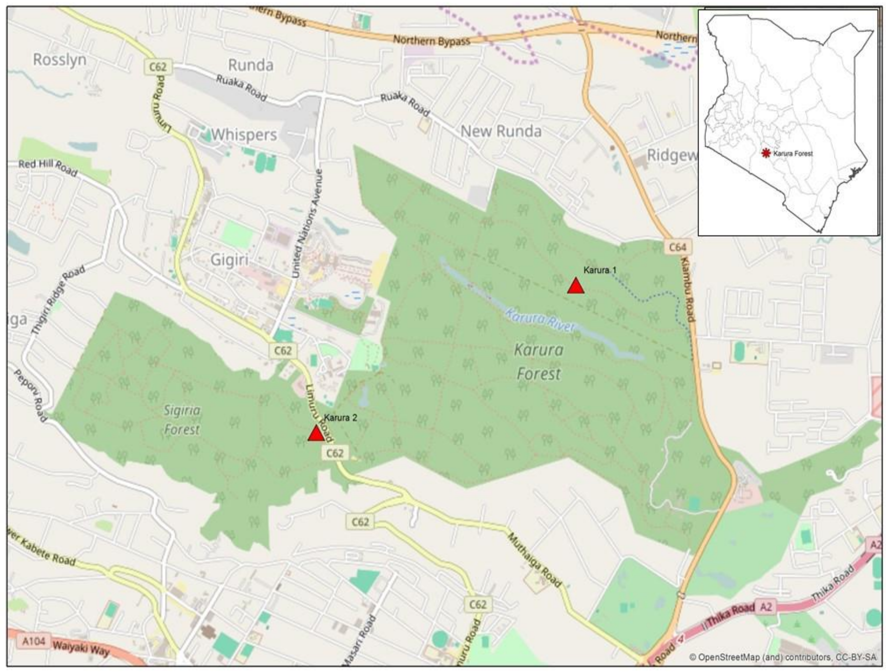

Figure 3.1: Location of the apiary sites at Karura Forest in Kenya. The two sites are marked in red, (Google Map).

Karura Forest is an urban forest in Nairobi, the capital of Kenya, located north of central Nairobi. The western part of the forest is also known as Sigiria Forest. The forest was gazetted in 1932 and is managed by the Kenya Forest Service under the Forest Act 2005, in conjunction with the Friends of Karura Forest. It is a key urban forest where the late Professor Wangari Mathaai a Nobel Laureate fought for its conservation in the late 1990s. It covers an area of 1041.3 hectares, making it the largest of the three main gazetted forests in Nairobi Arboretum and Ngong forests. Features in the forest include a waterfall, bamboo forest, marshland, Mau Mau caves and an old church (Alden & Mbaya, 2001). Rainfall ranges from 930 mm to 1250 mm while temperature ranges between 8^0c to 28^0c. The dominant vegetation cover around the apiaries is *Eucalyptus saligna (*Myrtaceae) and *Croton megalocarpus* (Euphorbiaceae).

3.3 Experimental design

Two apiaries at Karura forest were selected for this study. In each apiary, honeybee colonies were evaluated for honey production, hygienic behaviour, defensive behaviour, swarming tendency, colony buildup rate, capped brood and *Varroa* mite load. Each parameter had its rating scale and the overall individual Colony Performance Factor (CPF) recorded by adding the total scores. The colonies which had the highest scores were high in hygienic behaviour and low in *Varroa* mite load and the two were the main selection parameters for disease and pest's resistance, were selected as breeder colonies (Figure 3.2) (Boecking *et al*., 2000). One of the breeder colonies was used to provide the larvae for grafting (transfer of young larva from the natural worker cell to artificially

made cell cups), to minimize genotypic variation while another breeder colony was used to raise the drones used in artificial insemination. Twenty-four colonies of equivalent strength and mothered by one-year old queens were identified through selective sampling for raising the experimental grafts which were referred to as queen rearing colonies (Figure 3.2). Each graft had five replicates. Half of the colonies were randomly allocated for feeding regime of pollen supplement. The colonies were fed for four weeks before the grafts were introduced which continued until the grafts were sealed. The other set was not fed. Immediately after the queens hatched morphometric measurements were carried out.

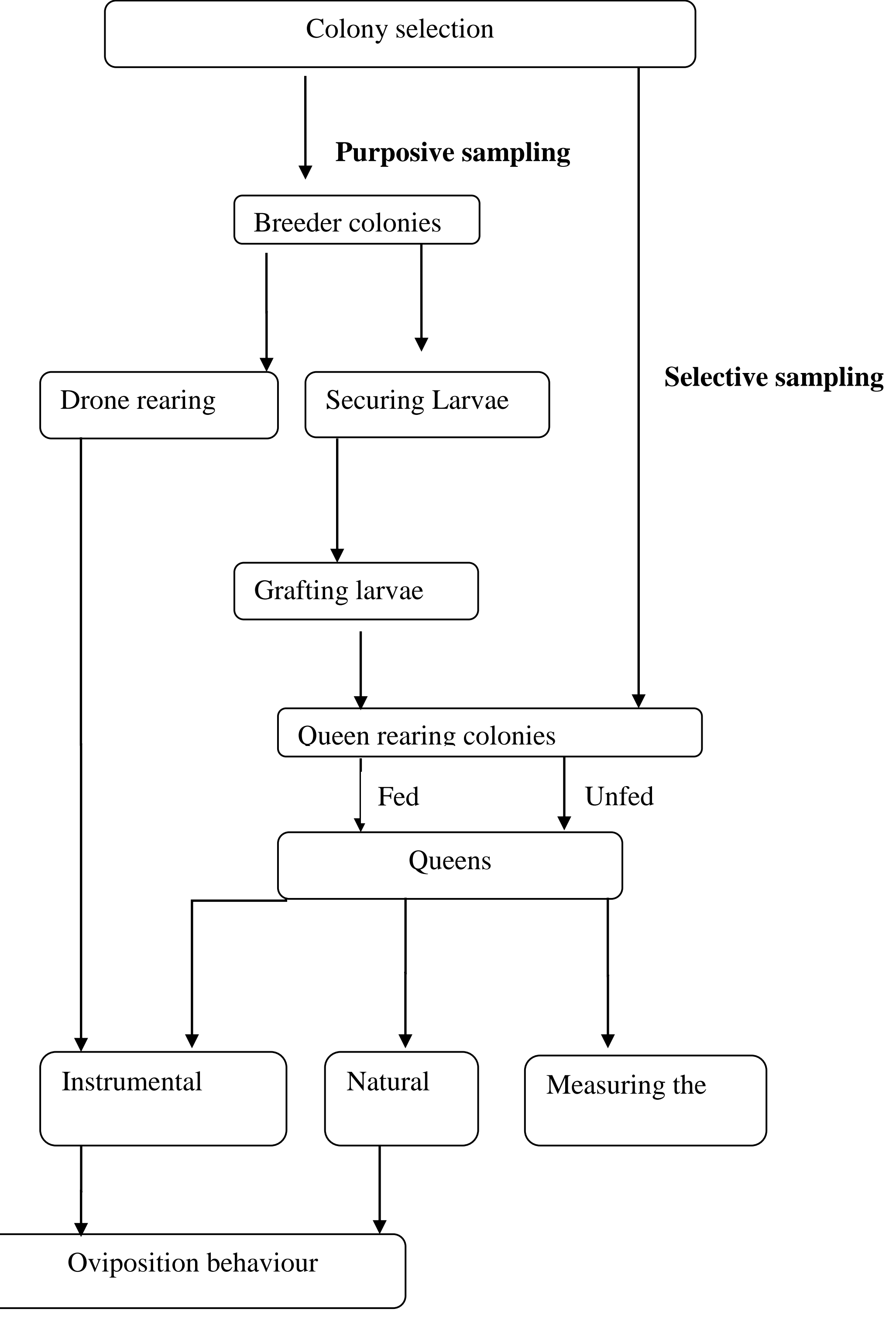

Figure 3.2: Schematic experimental design

3.4 Sampling design

The sampling method applied to identify the breeder colonies was non- probability purposive sampling design/judgmental (Figure 3.3). This was because after inspecting and evaluating the colonies for honey production, hygienic behaviour, defensive behaviour, swarming tendency, colony buildup rate, capped brood and *Varroa* mite load, a scoring system was used to select the colonies with the highest scores. The above-mentioned colony phenotypes were measured to select outstanding breeder colonies from the population rather than randomly choosing the colonies, which could result in undesirable physiognomies. These breeder colonies were then randomly selected for parentage. Since queen rearing colonies are required to be headed by a one-year old queen and of equivalent strength, selective sampling was employed in this case (Figure 3.3).

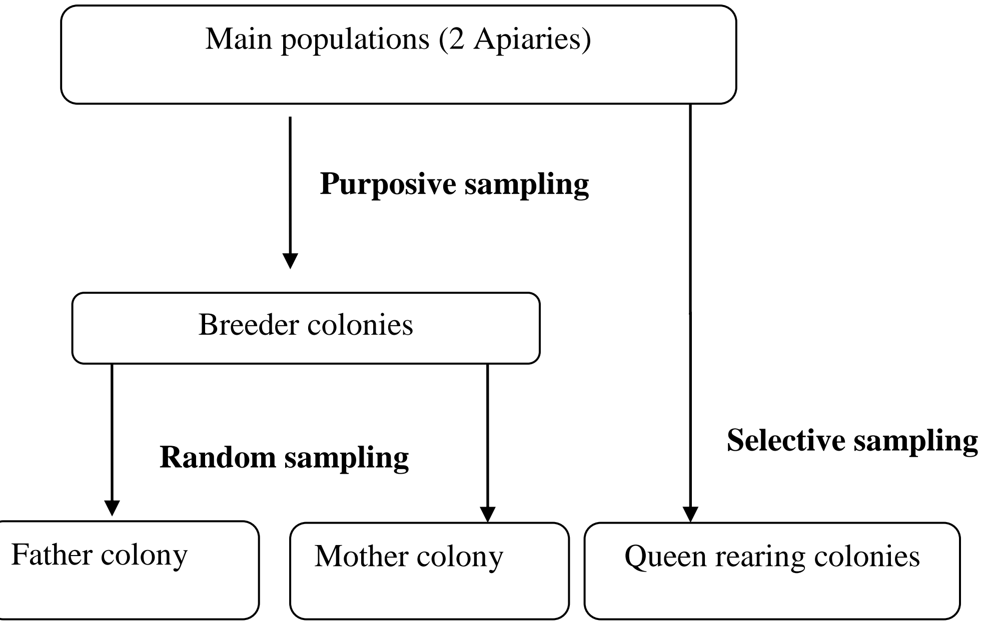

Figure 3.3: Schematic sampling design

3.4.1 Sample size determination

The sample size was determined by considering similar previous studies conducted by Mahbobi *et al*. (2012) on Iranian queen honeybees *Apis mellifera meda* and Gencer *et al*. (2000) on *Apis mellifera anatoliaca.* This led to the selection of 12 colonies used for queen rearing and 24 colonies which were used for artificial insemination.

3.5. Effects of larval age on morphometrics of Apis mellifera scutellata queens

A preliminary study was carried out to identify the breeder colonies which were confined in queen confinement cages to provide the larvae for grafting queens and raising drones for semen production in the experiment.

3.5.1 Evaluation of bee colonies for selection of the breeder stock

Thirty-four colonies at Karura forest ICIPE apiary 1 and 2 were inspected and evaluated for honey production, hygienic behaviour, defensive behaviour, swarming tendency, colony buildup rate, capped brood and *Varroa* mite load. A four-point scoring system for swarming tendency, colony buildup rate, capped brood defensive behaviour, infestation by *Varroa* mite and a five-point scoring for hygienic behavior were used to record the observations of these characteristics. This was done following methods described by Ruttner (1972). In purposive sampling, breeder colonies (mother and father) were drawn from the colonies with the highest score. The above-mentioned colony phenotypes were measured to select outstanding breeder colonies from the population rather than randomly choosing the colonies, which could result in undesirable physiognomies.

3.5.1.1 Hygienic behaviour

Hygienic behaviour was conducted according to Nicodemo (2013); Spivak and Reuter (1998) whereby a hollow cylinder (figure 3.4a) of 3-inch diameter (figure 3.4b) was used. Liquid nitrogen was poured (figure 3.4c) to freeze a circular section of sealed brood

(figure 3.4d). A five-point scale was used to score for hygienic behaviour 5 (>95%), 4 (90–95%), 3 (80–89%), 2 (70–79%), 1 (<70%). Approximately 211 cells within the 3-inch diameter cylinder were frozen. A frame with at least 3-inch diameter circle of sealed brood containing fewer unsealed cells within the circle was selected. The frame was laid horizontally across a support (figure 3.4a). The cylinder was twisted into the sealed brood until it reached the midrib (figure 3.4a). The number of unsealed cells inside the cylinder was recorded. Liquid nitrogen (300 - 400 ml) was poured into the cylinder and allowed to freeze the selected section (figure 3.4c). The cylinder was given three to five minutes to thaw and then removed. The frame was marked and returned into the hive. The frame containing the frozen brood was removed 24 hours (figure 3.4e), 48 hours (figure 3.4f) and 72 hours later (figure 3.4g) and the number of sealed cells, open and manipulated cells remaining within the circle recorded.

3.5.1.1.1 Schematic Hygienic behaviour, figures 3.4a to figure 3.4g

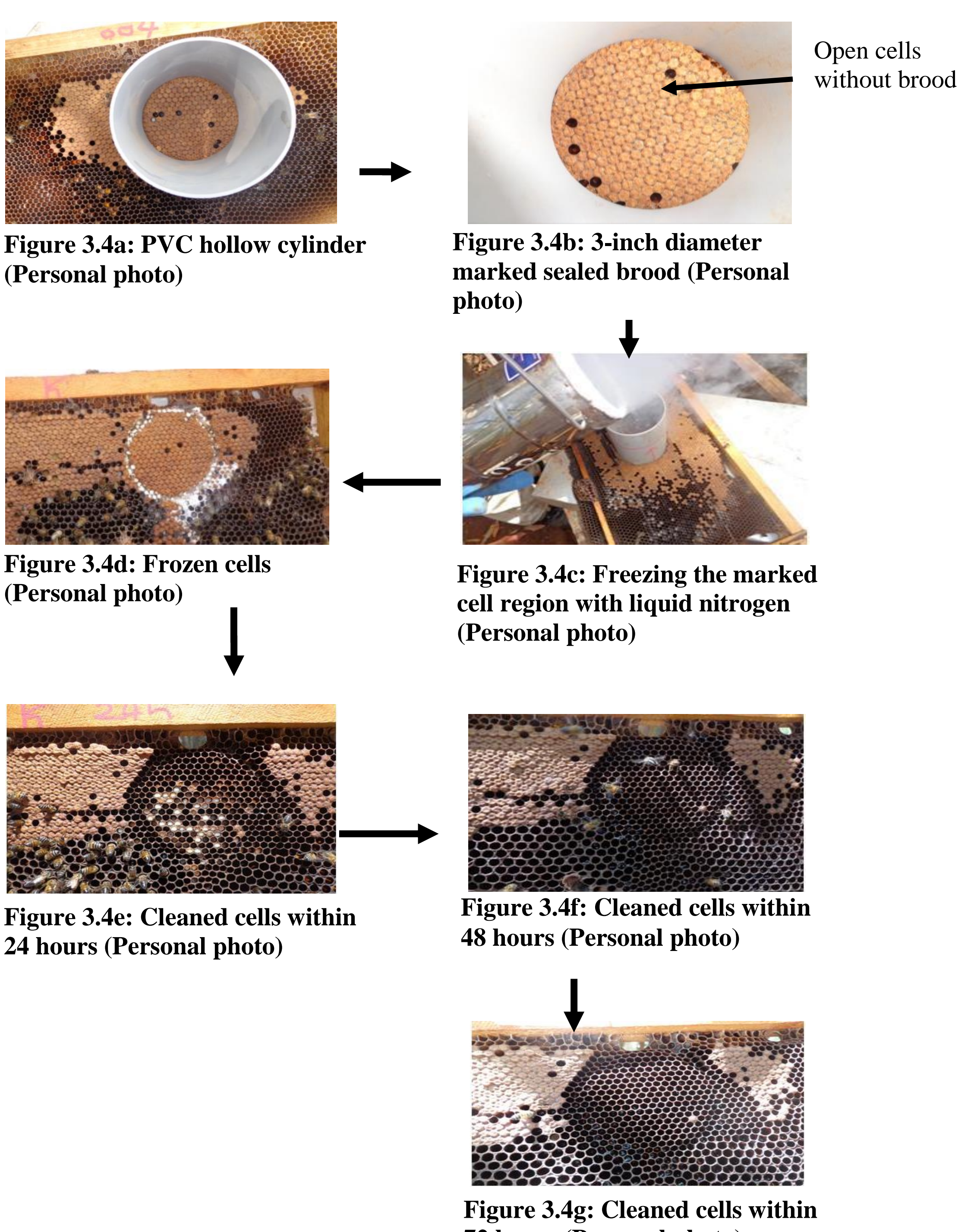

Figure 3.4a: PVC hollow cylinder (Personal photo)

Figure 3.4b: 3-inch diameter marked sealed brood (Personal photo)

Figure 3.4c: Freezing the marked cell region with liquid nitrogen (Personal photo)

Figure 3.4d: Frozen cells (Personal photo)

Figure 3.4e: Cleaned cells within 24 hours (Personal photo)

Figure 3.4f: Cleaned cells within 48 hours (Personal photo)

Figure 3.4g: Cleaned cells within 72 hours (Personal photo)

3.5.1.2 Varroa mite infestation

Sugar-shake method was used to estimate *Varroa* mite load (Ellis & Macedo, 2001). Live bee samples were obtained (figure 3.5a) so that the *Varroa* mites could be collected directly from the bodies of the bees by slightly shaking them in a mason jar containing icing sugar (figure 3.5b) that wound dislodge the mites on the bees (figure 3.5c). In this way, the entire colony infestation level was estimated. Infestation level was recorded using the rating 1- 4; high, moderate, low and zero. The percentage mite infestation was calculated by dividing the number of collected mites by the number of bees sampled (300) multiplied by 100.1% -3% low, 4% -9% moderate and 10% - 20% and above high (Dietemann *et al*., 2013).

Figure 3.5a: Using a Mason jar and a cup to measure 300 adult bees (Personal photo)

Figure 3.5b: A table spoonful of icing sugar added to the bees in the Mason jar then shaken to dislodge the mites from the bees (Personal photo)

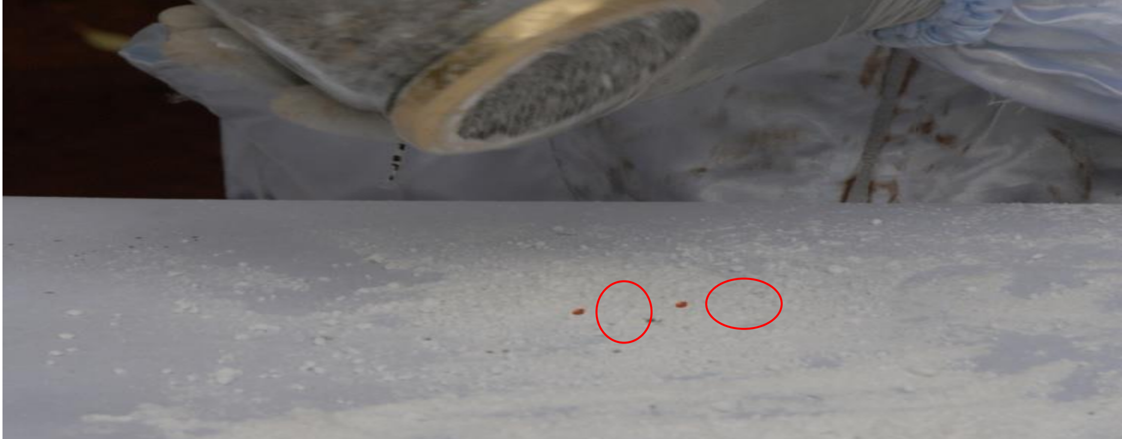

Figure 3.5c: Dislodged mites from the bees fallen on a white sheet of paper for counting (Personal photo)

3.5.1.3 Colony build up rate

Colony build up rate was evaluated following the method by Delaplane *et al.* (2013) whereby a subjective mode that relies on visual estimates by two observers was applied to quantify the following: (1) total occupied frames with brood (brood area), (2) total amount of honey, and (3) pollen intake.

The brood area was estimated by counting the number of combs containing brood. Brood on one side of the comb was counted as 0.5. A 4-point scoring protocol according to Delaplane *et al*., (2013) was followed.

4 points: brood present on more than 75 % of the comb

3 points: brood present on 50 – 75 % of the comb

2 points: brood present on 25-50 % of the comb

1 point: less than 25 % of the whole comb area is covered with brood

Honey productivity (storage test) was estimated by grouping 50 young bees of the same age in an observation cage at a temperature of 34ºC. The same quantity of 50% sugar syrup and protein was provided to each group and a piece of empty comb fixed in each cage. The population that stocks food products quickest formed the brood stock (Fert, 1996).

3.5.1.4 Swarming tendency

Ruttner (1972) method for monitoring swarming behavior in honeybee colonies was followed.

Table 3.1: Scoring criteria for swarming tendency

Points	Symptoms of swarming behaviour
4	No swarming tendency.
3	Low swarming tendency- There is some swarm cells in preparation for swarming.
2	Strong swarming tendency as indicated by repeated queen cell construction.
1	Active swarming indicated by the test colony having swarmed.

3.5.1.5 Defensive behaviour

Ruttner (1972) method for monitoring defensiveness in honeybee colonies was followed.

Table 3.2: Scoring criteria for defensive behaviour

Points	Gentleness	Calmness
4	No use of smoke and no protective clothes are necessary to avoid stings during normal working procedure	Bees stick to their combs, no notable reaction to being handled.
3	Colony can easily be worked without stings, if using some smoke.	Bees are moving, but do not leave their combs during treatment.
2	Single bees attack and sting during working procedure, even if smoke is used intensively	Bees partly leave their combs and cluster in the edges of frames and supers.
1	In spite of the use of smoke the colony shows a strong defensive reaction on being handled, or bees attack without being disturbed.	Bees nervously leave the combs, run out of the supers and cluster inside or outside the hive.

3.5.2 Queen rearing with different larval age groups

Five larval age groups, namely 6, 12, 24, 36 and 48 hour-old larvae were used (figure 3.6a). A queen from one of the breeder colonies was confined as shown in figure 3.6b to provide larvae of the right age for grafting starting with 48 hours.

Dark empty worker combs were supplied to the breeder colony in subsequent manner to suit the 48, 36, 24, 12 and 6-hour-old larvae prior to grafting.

The confinement procedure started at 1800hr of day 1 to 2200hr on the same day, to provide eggs for 48-hr-old larvae, and the frame containing the eggs labelled 48hr. On day 2, the queen was confined from 0600hr to 1000hr, to provide eggs for 36-hr-old larvae, and the frame containing the eggs were labelled 36hr. On the same day at 1800hr to 2200hr, the queen was confined to provide eggs for 24-hr-old larvae, and the frame labelled appropriately. On day 3 at 0600hr to 1000hr, the queen was confined to provide eggs for 12-hr-old larvae, and the frame labelled appropriately. On the same day, the queen was confined at 2400hr to 0400hr to provide eggs for 6-hr-old larvae, and grafting was done on the sixth day starting from day 1 of confinement at 1000hr.

Queens were reared using the Doolittle (1915) grafting method in 12 honeybee colonies of equivalent strength having 10 frames of bees mothered by one-year-old queens. The colonies were randomly assigned to two feeding regimens; fed with additional Pollen supplement (icing sugar, milk powder, and honey in the ratio of 2:2:1, respectively) and

not fed. Feeding was done four weeks before grafting, which continued until the queen cells were capped.

Beeswax was melted and cooled to 45ºC. Cell cups were then prepared by dipping the forming stick in cold water and then dipping it in melted wax then again in cold water and twisted to remove (Doolittle, 1915). Fixing on the grafting frame was done by the same wax. Twenty-five cells cups were fixed on each grafting frame with two grafting bars. The selected twenty-four queen rearing colonies of equal strength and headed by a one-year-old queen were used.

The colonies were rendered queenless for 24 hours before grafting. Cell cups were provided to the respective colonies 30 minutes before grafting for familiarization and cleaning.

After the 30 minutes were over, labelled frames with larvae of different ages were then taken out and grafting done using a grafting tool (figure 3.6c and figure 3.6d). Grafting was done concurrently from different frames containing different larval age groups. Each larvae age set was grafted into 5 labelled cells per grafting frame, and acceptance rate recorded. Each age set took 5 cells per frame. Larvae of different ages were grafted separately. One day before the estimated time of emergence of the virgin queens, capped queen cells in each experimental colony were confined using queen banks (figure 3.6e) and hair rollers (figure 3.6f) to protect the queens from attacking each other after emergence.

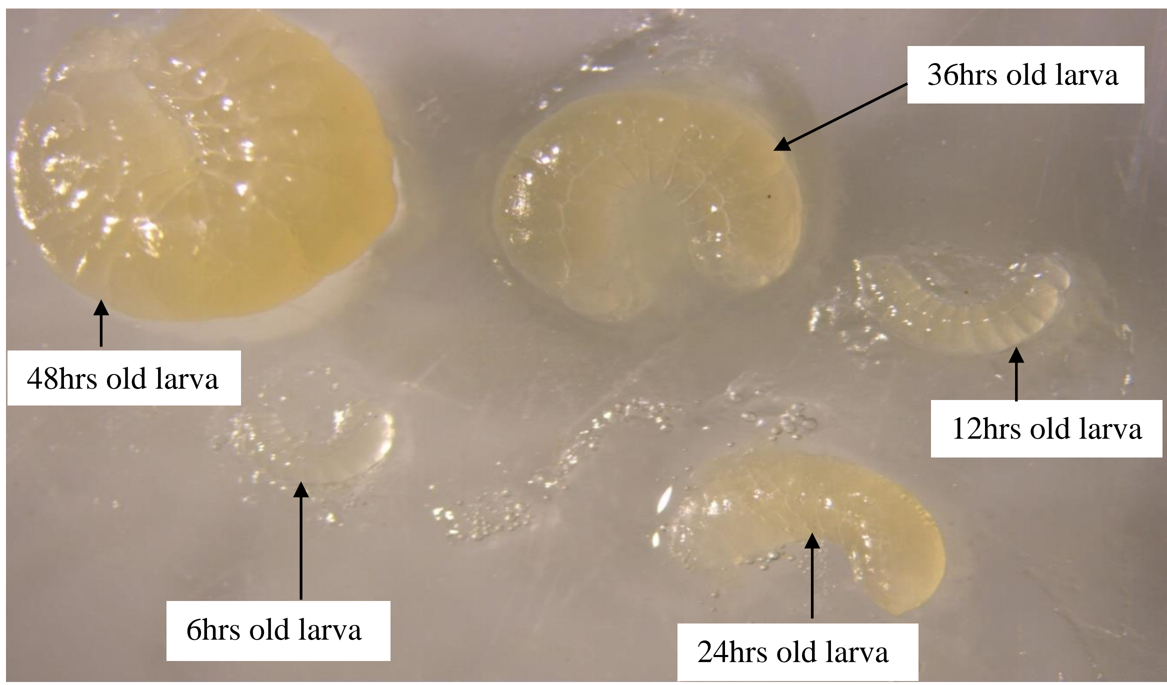

Figure 3.6a: Different larval age groups, 48-, 36-, 24-, 12- and 6 hrs (Personal photo)

Figure 3.6b: Confining the queen to secure right age larvae for grafting (Personal photo)

Figure 3.6c: Placing the grafted larva into the artificial bee's wax cell cup (Personal photo)

Figure 3.6d: Grafting (Personal photo)

Figure 3.6e: Confining the queen cells by queen bank (Personal photo)

Figure 3.6f: Confining the queen cells by hair roller (Personal photo)

3.5.3 Morphometric analysis of the external and internal body parts of newly emerged queens of *Apis mellifera scutellata*

The newly-emerged queens (figure 3.7a) were first immobilized at -20ºC for about 3 minutes, to record the wet weight using a digital scale to the nearest 0.1 mg, then immediately killed and the following morphometrics measured:

(1) External body characteristics such as head width (W) and head length (L) (figure 3.7b), thorax width and thorax length (figure 3.7c), wing width and wing length (figure 3.7d) and

(2) Internal body characteristics such as spermatheca length and width (figure 3.7e).

A Stemi 2000-C dissecting microscope with a Zeiss camera (Axiocam 105 color) (Carl Zeiss Microscopy GmbH 37081 GÖttingen, Germany) mounted on a computer and a monitor were used to measure the body parameters. The magnification used was lens 1.6x, camera 0.5x and zoom 0.65x = total magnification 0.52x. A pair of fine forceps was used to separate each individual part for measurement. To access the spermatheca, the queens' abdomens were dissected using a scalpel and pair of forceps. The measured length and width of spermatheca was used to calculate spermatheca volume (SV) according to the formula: SV = (4/3) (π) (r3), where r is the average of length and width of spermatheca (Hatch *et al.,* 1999). Spermatheca volume is an indicator of storage capacity of semen.

Figure 3.7b: Head length and width (Personal photo)

Figure 3.7a: Virgin queen emerging from the queen cell (Personal photo)

Figure 3.7d: Right wing length and width (Personal photo)

Figure 3.7c: Thorax length and width (Personal photo)

Figure 3.7e: Spermatheca length and width (Personal photo)

3.6 Effects of supplemental feeding on the morphometrics of *Apis mellifera scutellata queens*

The Twenty-four rearing colonies were randomly assigned to two feeding regimes; fed with additional pollen supplement and not fed. Half was fed (experimental) and the other half was not fed (control). Experimental colonies were fed twice a week on additional supplemental diet (pollen supplement) figure 3.8. Feeding was done four weeks before grafting and continued until the queen cells were capped on the 4th day after grafting. Floaters were placed on top of the food to prevent the bees from getting stuck in the food. Morphometric measurements were done for queens in both the experimental and the control groups.

Figure 3.8: Bees feeding on supplemental diet containing a mixture of milk meal powder, icing sugar and honey (Personal photo)

3.7 Evaluation of oviposition behaviour of naturally mated and artificially inseminated *Apis mellifera scutellata* queens

Queens were artificially inseminated while others were allowed to go for nuptial flights in order to study the oviposition behaviour.

3.7.1 Rearing of queens for artificial insemination and natural mating

Queens were reared from one of the breeder colony using larvae of ages capable of producing queens with best quality (12 - 24 hours old larvae) as derived in sub- section 3.5.1. Nucleus colonies were raised by dividing selected colonies and then introducing the developing queen cells (figures 3.9a and 3.9b) from the breeder colony on the 10th day of larvae grafting. A developing queen cell was introduced into each of the 24 nucleus colonies and the worker bees immediately started forming a retinue around the cell due to the queenless state of the nucleus colony (figures 3.9c and 3.9d). Twelve of the twenty-four nucleus colonies containing introduced queen cells had their entrances confined using pieces of queen excluders at the hive entrances to restrict the queens from nuptial flights. This group of queens was used for artificial insemination. The remaining twelve nucleus colonies with open entrances were the colonies in which virgin queens mated naturally during nuptial flights.

Figure 3.9a: Developing queen cells (Personal photo)

Figure 3.9b: Separation of queen cells for introduction into the nucleus colony (Personal photo)

Figure 3.9c: Introducing the queen cell on the face of a comb (Personal photo)

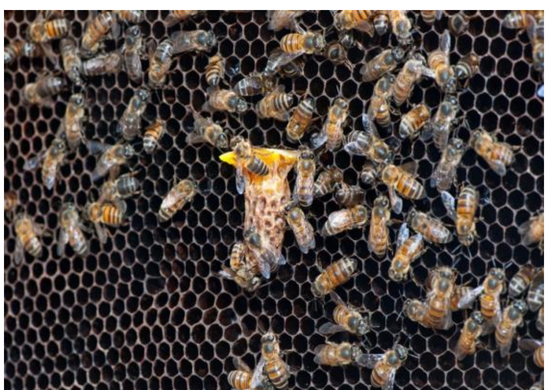

Figure 3.9d: Bees tending to an introduced queen cell because they are experiencing queenlessness (Personal photo)

3.7.2 Drone rearing

Drone rearing was done by looking for the queen in the breeder colony and confining it using a confinement cage and an empty drone comb. The queen then laid eggs in the empty drone comb and then it was released. The drone brood was left to cap and then confined above the queen excluder until was hatched. Once the drones emerged, the opening of the hive was controlled by opening it in the early morning to avoid drifting (Rhodes, 2002).

3.7.2.1 Transporting the drones and queens to the laboratory

Forty day's old mature drones were collected from the drone breeder colony early in the morning in a drone transportation cage. A small piece of comb honey was put in a small tray and put inside the cage for feeding the drones. Individual virgin queens were put in queen cages and the cage labelled according to the hive number. Five escort bees were put in the cages to feed the queens on transit.

3.7.2.2 Harvesting the semen

Semen collection was carried out using the methods described by Collins (2000) and Cobey *et al*. (2013) (figure 3.10a). Mature drones were squeezed at the thorax to achieve complete eversion of the endophallus to expose the semen. Semen located at the tip of the endophallus was collected using the capillary containing a buffer solution.

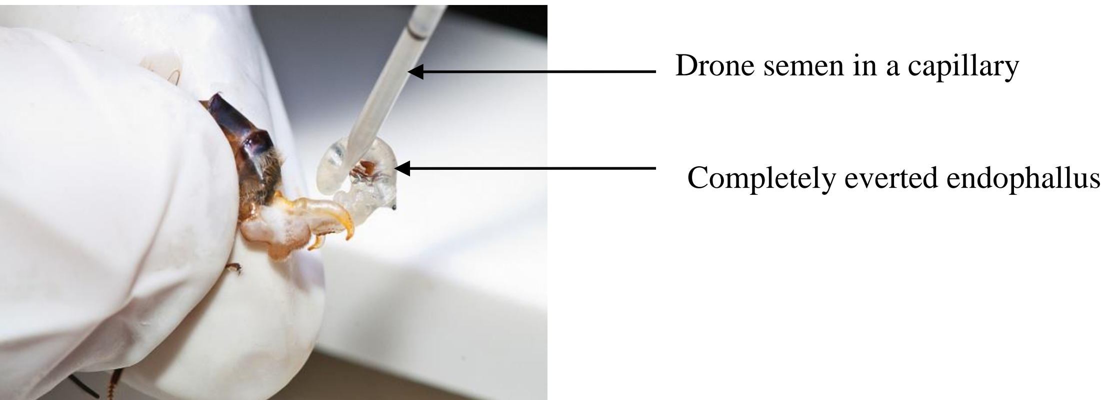

Figure 3.10a: Drone squeezed at the thorax to expose the endophalus for semen collection using a capillary (Personal photo)

3.7.2.3 Insemination

Twelve virgin queens were given preparatory carbon dioxide treatment for 7 minutes on day 6 of their emergence. They were then returned to their respective colonies awaiting insemination the next day. Each queen was inseminated on the 7th day (figure 3.10b). with 8 μl of semen and put back in the queen cage together with escort bees and given 10 minutes to revive (Collins, 2000; Cobey, *et al*., 2013). The queens were then re-introduced into their respective colonies in a queen cage (figure 3.10c) using candy prepared by mixing honey and icing sugar. Worker bees were attracted to the pheromone (queen substance) produced by the inseminated queen and they formed a retinue around the cage housing her (figure 3.10d).

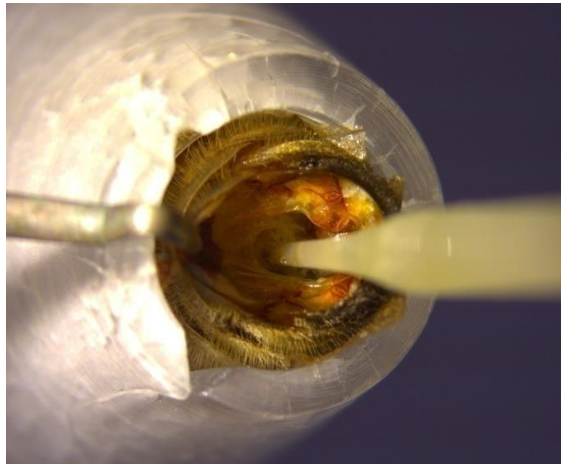

Figure 3.10b: Ventral and dorsal hooks stretches open queens' genital cavity during semen injection, magnification 0.52× (Personal photo)

Figure 3.10c: Introducing the inseminated queen back in the colony (Personal photo)

3.7.3 Monitoring for oviposition

The queens were monitored every 24 hours, to determine the first egg-laying date and the number of eggs laid by providing empty combs as laying spaces for the queens. Egg counting was done daily for 14 days until both inseminated and naturally mated queens increased their egg numbers. Counting continued biweekly and monthly using a transparent sheet of paper for both inseminated and naturally mated queens. A comb containing newly laid eggs was taken out and laid horizontally on a flat surface. A transparent sheet was placed on top, and using a marker pen, the cells with eggs were circled and then counted later; one sheet for side 'A' and another one for side 'B' of a single comb, then the total was calculated. The queen was then given another empty comb and if there was more space in the previous comb, the areas with eggs were marked with a marker pen and the comb left for the queen to lay again.

3.8 Data analysis

Data collection for objectives (i) and (ii) was simultaneous and the experiments occurred simultaneously. The proportion data on queen's acceptance were analyzed using generalized linear model (GLM) with logit link and binomial distribution error to evaluate the effect of larvae age and feeding group. Effect of factors for a GLM is reflected in the deviance that has an approximate chi-square distribution; hence, the chi-square values are presented as test statistics. Means were separated using adjusted Tukey, implemented using the glht function of the multcomp package (Hothorn *et al.*, 2008).

Multivariate analysis of variance (MANOVA) was used, to evaluate the effect of age and supplemental feeding on queen quality (fed and unfed groups of colonies) for all nine measured morphometrics simultaneously on each of the four larvae age groups (36, 24, 12 and 6-hours). Univariate analysis (one-way ANOVA) followed by SNK (Student Newman Keuls) post hoc test were used to further compare means of the individual morphometrics within each group where MANOVA (λ) was significant.

Data collected for objective (iii) was analysed using Student's t-test to determine if the two sets of data for naturally mated and artificially inseminated queens were significantly different from each other.

CHAPTER FOUR

RESULTS

4.1 Effect of larval age on the morphometrics of Apis mellifera scutellata queens

4.1.1 Effects of larval age on acceptance

The acceptance rates of grafted larvae by queen rearing colonies are recorded in figure 4.1

Analysis of proportion data of grafted larvae acceptance showed that age affected *A. m. scutellata* queen acceptance rate independently ($\chi^2 = 0.35$, df = 4, p = 0.99). The main effect of larval age was significant ($\chi^2 = 142.53$, df = 4, $p < 0.0001$). Larvae grafted at age 6 and 48 hours had a lower acceptance rate compared to ages 12, 24, and 36 hours which showed no significant difference (Figure 4.1).

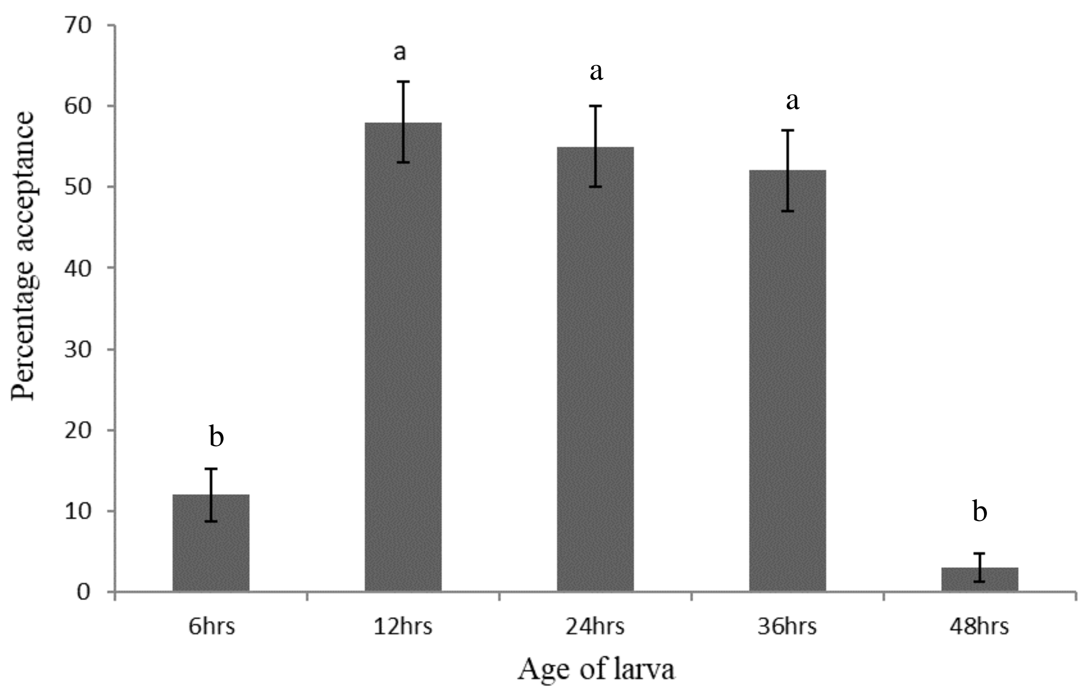

Figure 4.1: Grafted larvae acceptance rate expressed as percentage larvae grafted for the various larvae age groups.

4.1.2 Morphometric variability of queens reared from various larval ages

Results on the morphometric analysis of queens obtained from the grafting of five different larvae age groups, 48, 36, 24, 12, and 6 hours revealed significant differences in the queens reared from the fed group (Tables 4.1). There was no significance difference in head width between ages 12,24 and 36 but significant difference was noted in head length where age 24 was significantly higher. The thorax width was constant across the age groups. Thorax length of age 24 was statistically higher than thorax length of age 12 but there was no significant difference in the thorax length of ages 6 and 36 hours, which

also shown no significant difference with the thorax length of age 12 hours. The difference in ages of the grafted larvae within the fed group of experimental colonies significantly affected the queen characteristics (Table 4.1).

The 48 hours was excluded from the analysis due to its poor acceptance. The SNK post hoc test revealed that queens reared from 12- and 24-hour-old larvae were on average heavier and larger in most morphometric measurements than those reared from larval ages 6 and 36. The mean values of the wet weight of the queens reared from 24 hours old larvae were significantly heavier than the queens reared from the other age groups, 6 ,12 and 36 hours old larvae within the fed group (Table 4.1 and fig 4.2a).There was no statistical difference in the wet weight within the unfed group (Table 4.2 and Figure 4.2a).

The means of the spermatheca volume of the queens reared from ages group 12, 24 and 36 hours old larvae of the fed group were significantly higher than that of queens reared from 6 hours old in the fed group (Tables 4.1 and fig 4.2b). The means of the spermatheca volume of the queens reared from age group 12 an, 24 hours old larvae of the unfed group were significantly higher than the queens reared from 6 and 36 (Tables 4.2 and fig 4.2b). Queens reared from 12 and 6 hours old larvae were not significantly different in their wet weights but were significantly heavier than those reared from 36 hours old within the fed group (Table 4.1 and fig 4.2a). The internal morphometric parameter (spermatheca volume) varied in queens reared from the different larvae age groups. Larvae ages 12, 24 and 36 produced queens with larger spermatheca compared to

larvae age 6 (fig 4.2b). The only constant parameter across groups was the thorax width (Table 4.1). the other age groups, 6 ,12 and 36 hours old larvae within the fed group (Table 4.1 and fig 4.2a). There was no statistical difference in the wet weight within the unfed group (Table 4.2 and Figure 4.2a)

Table 4.1: Mean ± SE (n=102) of measured morphometrics of queen honeybees grafted from different larvae ages from fed colonies

Parameters	Larval age in hours			
	6	12	24	36
Wet weight (mg)	0.17±0.020[b]	0.16±0.020[b]	0.18±0.009[a]	0.14±0.018[c]
Head width (mm)	1.78±0.154[b]	1.87±0.065[a]	1.88±0.065[a]	1.86±0.076[a]
Head length (mm)	1.84±0.185[b]	1.81±0.210[b]	1.97±0.062[a]	1.84±0.117[b]
Thorax width (mm)	2.33±0.016[a]	2.30±0.220[a]	2.30±0.151[a]	2.30±0.151[a]
Thorax length (mm)	2.82±0.015[ab]	2.71±0.214[b]	2.88±0.106[a]	2.75±0.209[ab]
Wing width (mm)	1.65±0.010[a]	1.61±0.062[a]	1.51±0.088[b]	1.62±0.108[a]
Wing length (mm)	5.02±0.232[a]	4.86±0.276[ab]	4.70±0.117[b]	4.77±0.280[b]
Spermatheca volume (mm^3)	0.74±0.054[b]	1.02±0.033[a]	0.94±0.023[a]	0.94±0.024[a]

Mean separation is based on univariate analysis of variance. Means followed by the same letter within a row are not significantly different (Student–Neuman–Keuls test, α = 0.05).

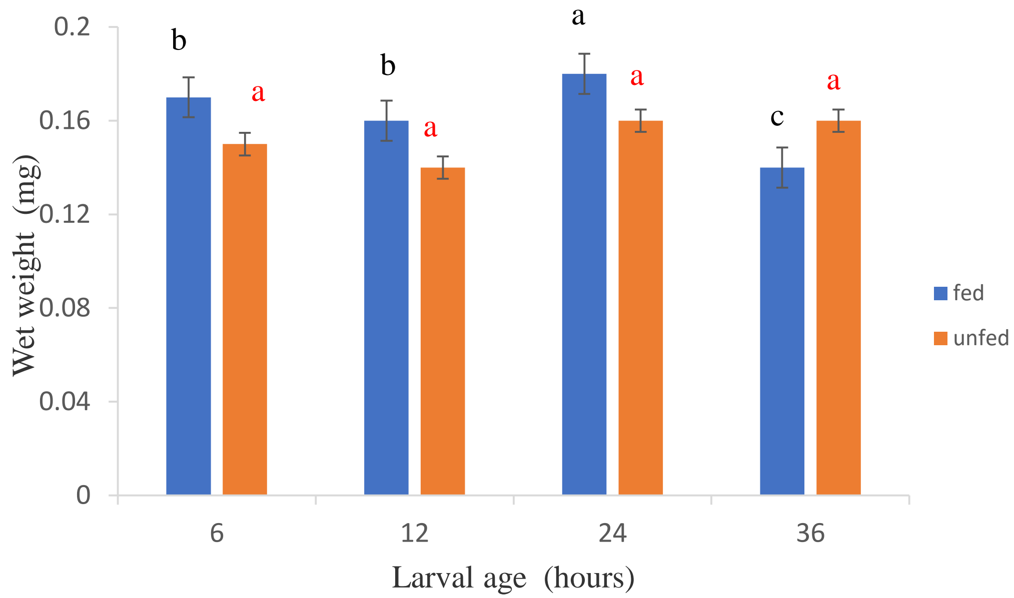

Figure 4.2a: Mean ± SE of wet weight in the different larval age groups of fed and unfed

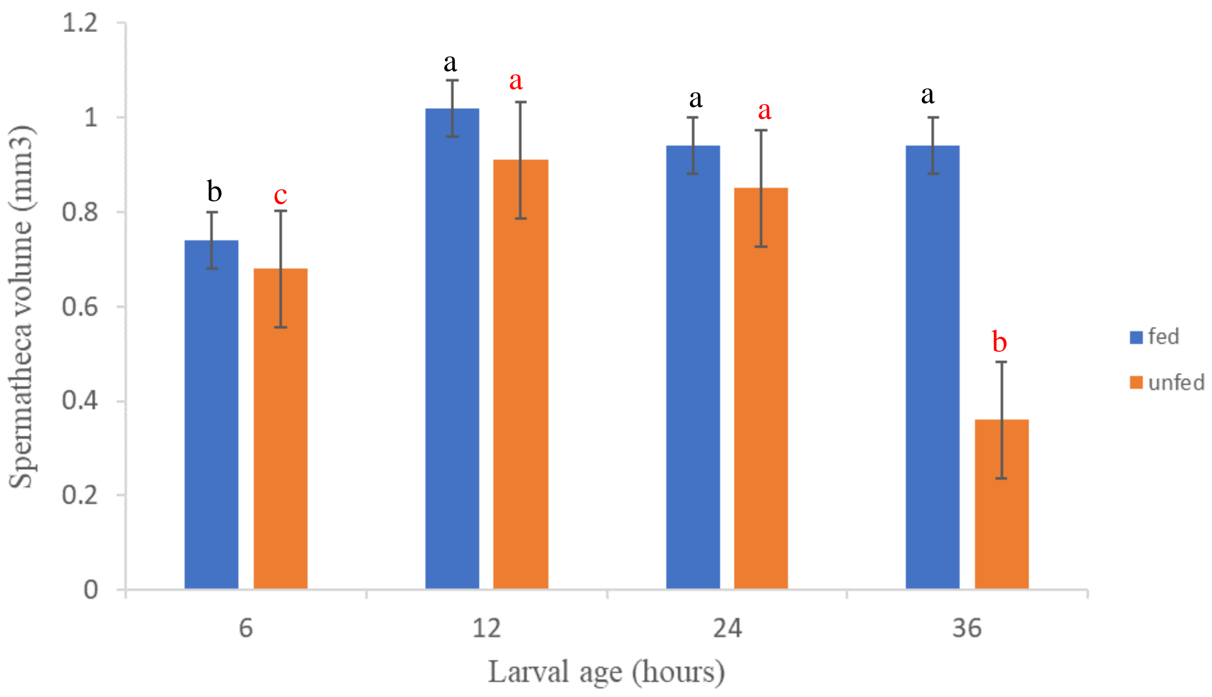

Figure 4.2b: Mean ± SE of spermatheca volume in the various larval age groups of fed and unfed groups

Table 4.2: Mean ± SE (n=78) of measured morphometrics of queens grafted from different larvae ages from unfed colonies

Parameters	Larval age in hours			
	6	12	24	36
Wet weight (mg)	0.15±0.008[a]	0.14±0.019[a]	0.16±0.032[a]	0.16±0.005[a]
Head width (mm)	1.72±0.146[b]	1.85±0.075[a]	1.80±0.054[a]	1.85±0.056[a]
Head length (mm)	1.79±0.185[a]	1.77±0.215[a]	1.88±0.105[a]	1.80±0.229[a]
Thorax width (mm)	2.33±0.018[a]	2.28±0.269[a]	2.19±0.217[a]	1.93±0.067[b]
Thorax length (mm)	2.82±0.016[a]	2.64±0.230[a]	2.68±0.213[a]	2.39±0.283[b]
Wing width (mm)	1.64±0.013[a]	1.57±0.072[a]	1.58±0.113[a]	1.65±0.056[a]
Wing length (mm)	4.97±0.251[a]	4.82±0.218[a]	4.79±0.190[a]	4.91±0.069[a]
Spermatheca volume (mm^3)	0.68±0.071c	0.91±0.037a	0.85±0.026a	0.36±0.013b

Mean separation is based on univariate analysis of variance. Means followed by the same letter within a row are not significantly different (Student–Neuman–Keuls test, α = 0.05).

4.2 Effects of supplemental feeding on the morphometrics of *Apis mellifera scutellata* queens

4.2.1 Effects of supplemental feeding on queen acceptance

Results on the analysis of proportion data on queen acceptance showed that age and supplemental feeding affected *A. m. scutellata* queen acceptance rate independently (χ^2 = 0.35, df = 4, p = 0.99). However, the main effect of supplemental feeding was significant (χ^2 = 5.01, df = 1, p = 0.025). The results of this study showed clear difference in acceptance rate between fed and unfed colonies (Figure 4.3a)

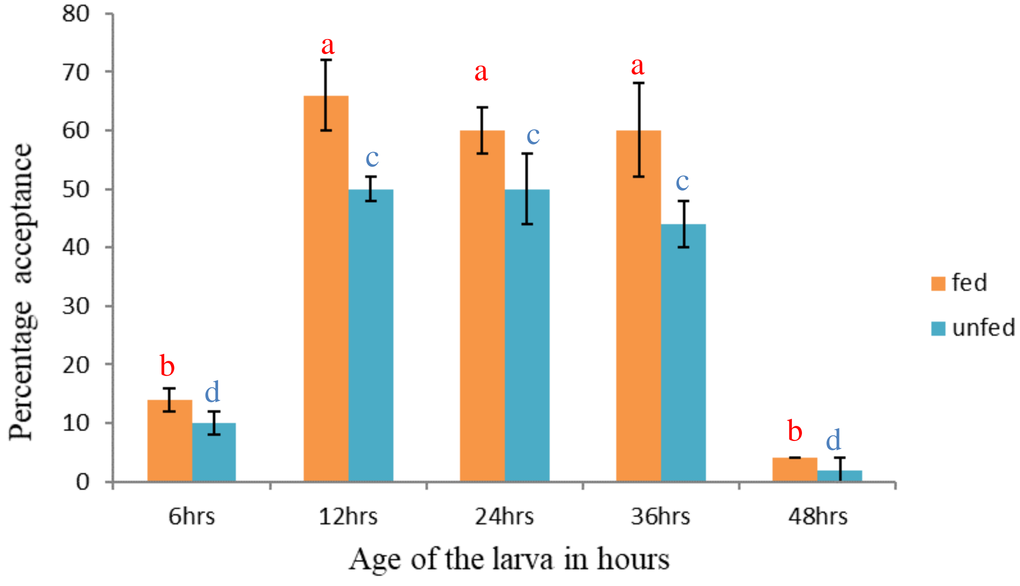

Figure 4.3a: Grafted larvae acceptance rate expressed as percentage larvae grafted for the fed and unfed groups

Colonies fed with supplemental diet had higher acceptance rate (41%) compared to unfed colonies (31%) fig, 4.3b.

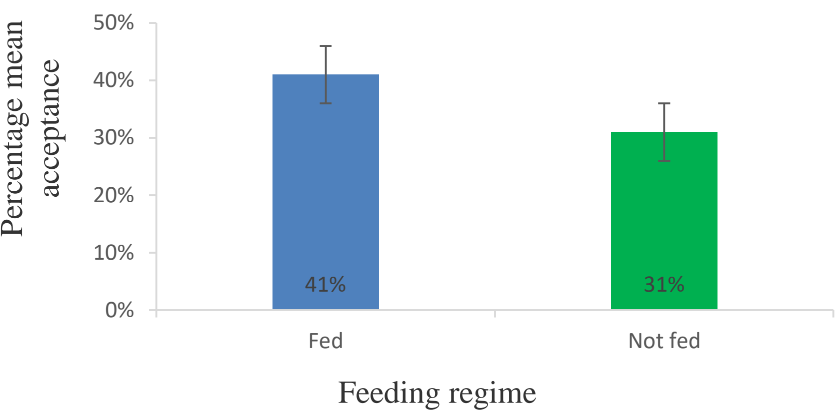

Figure 4.3b: Grafted larvae acceptance expressed as mean percentage for the five-age group

4.2.2 Morphometric variability of queens reared from fed and unfed groups of colonies

Comparison of morphometric parameters of queens reared from different larvae ages within the unfed groups of colonies produced different results. Multivariate analysis of the overall results indicated a significant difference between the two major classes of colonies (fed and unfed ($p < 0.001$).

The wet weight, head width and head length, thorax width and thorax length, spermatheca width and spermatheca length of fed group of colonies were significantly higher than those of the unfed group of colonies. However, the wing width and length of the two groups fed and unfed were not significantly different (Table 4.3). In the unfed group, the queens were significantly smaller than in the fed counterpart (Table 4.3). The spermatheca volume was significantly larger in queens reared from fed than those from unfed colonies (Table 4.3).

Table 4.3: Comparison of Mean ± SE of measured parameters of queens produced from fed and unfed groups of colonies

Parameter	Mean ± S.E. of fed queens (n=102)	Mean ± S.E. of unfed queens (n=78)	t-value	p-value
Wet weight (mg)	0.163±0.002	0.154±0.003	2.68	0.008*
Head width (mm)	1.862±0.008	1.828±0.009	2.87	0.005*
Head length (mm)	1.873±0.016	1.812±0.022	2.26	0.025*
Thorax width (mm)	2.264±0.002	2.151±0.029	3.29	0.001*
Thorax length(mm)	2.779±0.023	2.591±0.032	5.47	0.000*
Wing width(mm)	1.585±0.010	1.601±0.010	1.11	0.270
Wing length(mm)	4.795±0.025	4.843±0.021	1.40	0.162
Spermatheca volume (mm^3)	0.950±0.017	0.717±0.031	7.10	0.000*

4.3 Oviposition behaviour of Naturally Mated(NM) and Artificially Inseminated(AI) queens of *Apis mellifera scutellata*

Oviposition behaviour varied in both naturally mated and artificially inseminated queens with difference being recorded on onset and rates of oviposition.

4.3.1 Oviposition rates in naturally mated and artificially inseminated queens

All the queens emerged on day zero (figure 4.4a). The virgin queens assigned for artificial insemination had their hive entrances confined in their respective nuclear colonies while the ones assigned for natural mating their hive entrances remained open and they were able to perform their orientation flights between day 1 and day 5 then later for went nuptial flight. Naturally mated queens started laying eggs earlier (on the 6th day of their life) relative to the artificially inseminated queens (on the 11th day of their life) (Figure 4.4a).

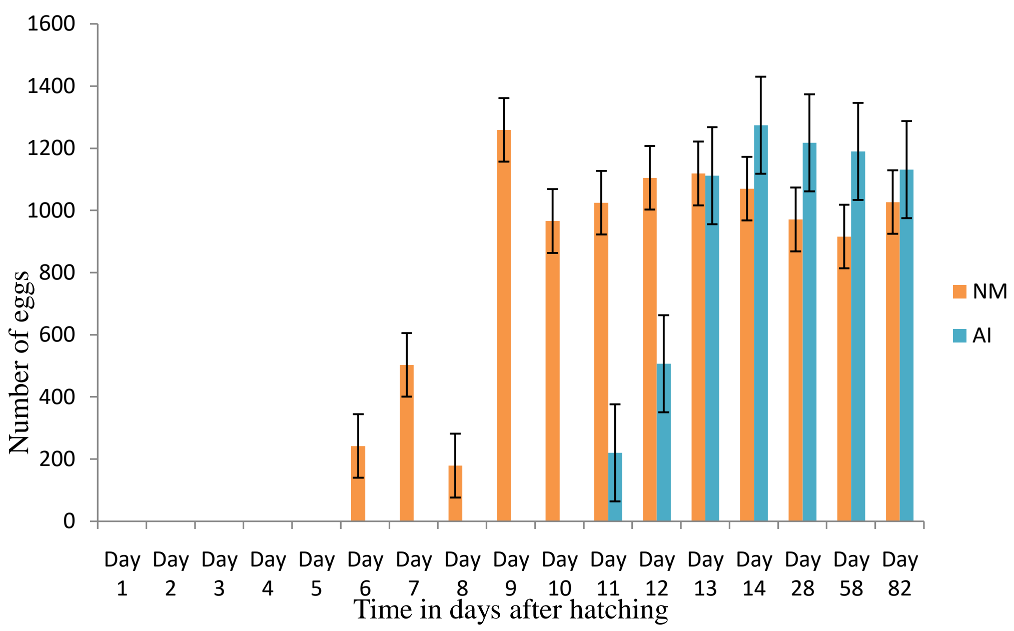

Figure 4.4a: Oviposition behaviour in naturally mated and artificially inseminated *Apis mellifera scutellata* queens

In addition, naturally mated queens laid slightly more eggs on the first egg-laying day compared to artificially inseminated queens. The onset of oviposition was relatively low in both queens in the first three days (Figure 4.4b). On the second day of egg-laying, oviposition was levelled up for both inseminated- and naturally-mated queens (Figure 4.4b). Eggs laid by AI queens on day 3 were significantly higher (figure 4.4b).

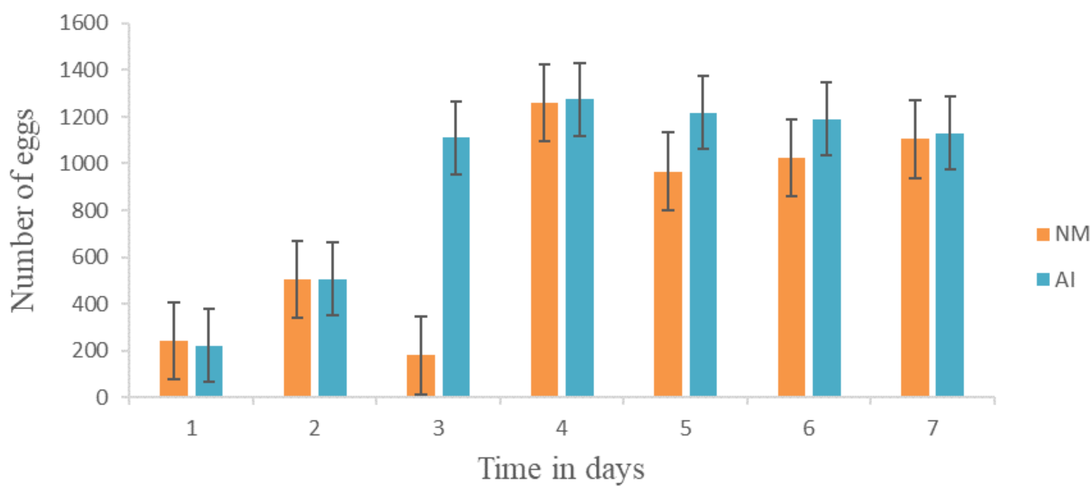

Figure 4.4b: Mean number of eggs in Naturally-Mated (NM) and Artificially-Inseminated (AI) *Apis mellifera scutellata* queens

Artificially inseminated queens laid a single egg per cell and vertically placed at the bottom of the cell (figure 4.4c) as applied to the naturally mated queens (figure 4.4d).

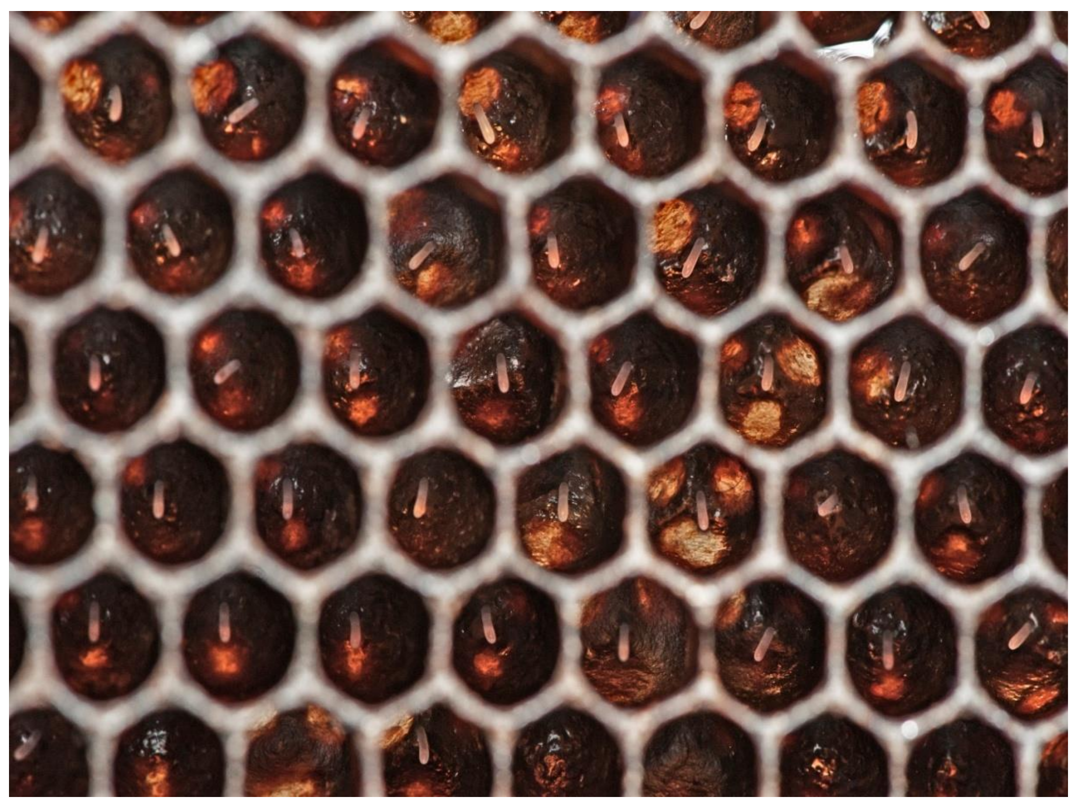

Figure 4.4c: Eggs laid by an artificially inseminated queen (Personal photo)

The egg laying pattern was found to be uniform and displayed a good and systematic arrangement in the comb for both artificially inseminated and naturally mated queens (figure 4.4c and figure 4.4d).

Figure 4.4d: Eggs laid by a naturally mated queen (Personal photo)

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Effect of larval age on acceptance

The higher acceptance rate obtained for larvae ages 12, 24 and 36 h within both fed and unfed groups indicates that these larvae ages are suitable for grafting *A. m. scutellata* queens irrespective of feeding scheme. The low acceptance rates in the two ages, age 48 and 6 hours old larvae was due to their sizes during grafting. Age 48 hours larva were too large, and they had consumed almost all the royal jelly (food) supplied to it by the nurse bees, and age 6 hours larva were too small and had little royal jelly supplied to them by the nurse bees because they had just hatched a few hours ago. During grafting the larva should be amply supplied with royal jelly and floating on top of it to ensure that the larva is not touched by the grafting needle since they are very delicate, and to minimize on the larva drying up during the transfer process. This finding is consistent with reports by Muli *et al*. (2005) on *A. m. scutellata* and *A. m. monticola* larvae that cell acceptance rates were highest in the 24-h-old larvae (74.5%) and least in 48- and 60-h-old larvae (35%), but inconsistent with the acceptance rates reported for *A. m. anatoliaca* that 2-day- old larva were readily accepted than 1 -day- old larva (Gencer *et al*., 2000). This study showed that the acceptance rate was affected by larval age as opposed to reports by

Gencer *et al*. (2000) where they did not find any relationship between larva age and acceptance.

5.1.2 Effects of larval age at grafting on the morphometrics of the honeybee queens of *Apis mellifera scutellata* queens

The wet weight of the virgin queens after emergence was influenced by larval at grafting. The weight of queens grafted from all the accepted larval ages (6, 12, 24, and 36-h-old) indicated that all the colonies were productive and of equal strength which is consistent to (Souza *et al.,* 2013; Delaney *et al*., 2011; Kahya *et al*., 2008) who reported that the body weight of the queen honeybee serve as a potential indicator of colony productivity. However, within the fed group, the 24-h-old larvae had heavier queens. Queens grafted from the 12 and 24 hours old larvae were heavier and larger in most morphometric parameters.

Larger spermatheca volume was reported in queens reared from the 12- and 24-hr-old larvae from within the fed and unfed colonies. therefore, suggests that these two age groups are best for rearing *A. m. scutellata* queens since it agrees with (Carreck *et al*., 2013) suggestion that the quality of a queen and thus the colony, depends on the spermatheca size and volume. Even though the 6- and 48-hr-old larvae produced queens with high wet weights, their low acceptance and spermatheca size makes them less suitable for grafting regarding queen rearing.

5.1.3 Effects of supplemental feeding on the morphometrics of *Apis mellifera scutellata* queens

The study demonstrated that additional supplemental feeding affected most morphometric characters of the reared queens of *A. m. scutellata*. This supports findings by Mahbobi *et al*. (2012) for the Iranian honeybee *A. m. meda* queens that supplemental feeding significantly

affects most of the morphological. The interaction between age and feeding implies that supplemental feeding may affect the weight of the resultant queens of some larvae age 24 hours more than others. This is an indication that nutrition is very essential in the queen development and quality, especially when there is no pollen supply in the surrounding. Earlier studies by (Hussein *et al*., 2000; Gençer *et al*., 2000), have demonstrated the importance of artificial supplementation such as syrup, pollen and vitamins or carbohydrates and proteins on queen rearing and beekeeping in general. This is because when this work was carried out, the pollen intake was minimal in the colonies therefore the pollen supplement was necessary and played a crucial role in the process. The less feeding of the queens in the unfed colonies resulted into light weight queens and smaller spermatheca.

5.1.4 Oviposition behaviour of naturally mated and artificially inseminated queens of *scutellata*

The Orientation flights by naturally mated queens took place within the first five days of their life. This does not support the reports by (Woyke, 2008) that queens do not start mating flights before the age of six days.

The onset of oviposition in naturally mated queens commenced earlier than the artificially inseminated queens. This is probably because of the difference in queen treatment which might determine the duration taken by the sperms to migrate from the genital tract to the spermatheca. These results confirm previous reports which indicated that artificially inseminated honeybee queens initiate oviposition much later than naturally mated queens (Woyke, 2008). The non-significant difference in the number of eggs laid by AI and NM supports findings by (Cobey, 2007) that the naturally mated and artificially inseminated queens have equal performance. Nevertheless, given that there is no selection of traits in NM as opposed to AI queens, artificial insemination remains the best method to control mating and selection of desired traits.

5.2 Conclusions

The study concludes that:

i. The best age for grafting *A.m. scutellata* queens is 12 and 24 hours old larvae

ii. Supplemental feeding is an important factor that can improve the production of high quality *A.m. scutellata* queens.

iii. Artificially insemination technique is important in breeding programmes due to the advantage of controlled mating and thus can be adopted for queen rearing.

5.3 Recommendations

i. This study has shown that artificial insemination does not interfere with egg laying and can be used in controlled mating in *A. m. scutellata*. It is therefore, recommended that this technique be used to produce queens of superior quality for improving pollination and hive product.

ii. Artificial insemination technique can therefore be used to further research on the production of high quality queens of *A. m. scutellata* through queen rearing or breeding programs.

iii. Further studies are needed to establish the correlation between the quality of the queens produced from the different age groups and the overall colony productivity.

iv. Honeybee colonies were not assessed for other pests other than *Varroa* mites and diseases during breeder colony selection. Further studies are needed to determine the effect of the age of grafted larvae and supplemental feeding on colony fitness.

v. AI and NM queens were not assessed further for honey productivity. Further studies are necessary to determine the production potential in both AI and NM queen's colonies.

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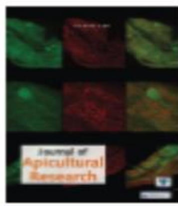
APPENDICES

Appendix 1: Parameters scored during the breeder colony selection and the overall colony performance factor.

Hive code	Hygienic behaviour (1 – 5)	Swarming behaviour (1- 4)	Colony Strength (1 -4)	Honey yield (1 -4)	Capped brood (1 -4)	Defensive behaviour (1 -4)	*Varroa* mite load (1 – 4)	CPF Max (0-29)
1	5	4	4	4	4	3	3	27
2	5	4	4	4	4	3	3	27
3	5	4	4	4	2	2	3.5	25.5
4	5	4	3	3.5	3	3	3	24.5
5	4	4	3	3	4	1	3	22
6	2	4	3.5	3	4	2	3	21.5
7	1	4	4	4	4	2	2	21
8	4	4	3	2.5	3	1	3	20.5
9	1	4	3.5	3	3	2	2	20
10	3	4	4	4	2	2	3	20
11	4	4	3	3	2	1	3	20
12	4	4	2.5	2	2	2	3	19.5
13	1	4	4	2	4	1.5	3	19.5
14	2	1	3	4	4	2	3	19
15	2	4	3	3	2	1	3	19
16	3	4	3	3	2	3	2	19
17	3	4	3	3	2	2	2	19
18	3	4	4	3	3	1	1	19
19	1	4	3.5	4	2	2	2	18.5
20	1	4	3.5	3	3	2	2	18.5
21	4	4	3	3	2	1.5	1	18.5
22	3	4	4	3	2	1	1	18
23	2	4	2	2	3	2	3	18
24	1	4	3.5	1	4	2	3	17.5
25	2	4	2	1	2	3	3	17
26	3	4	3	3	2	1	1	17
27	1	1	4	3	3	2	2	16
28	2	1	3	4	2	2	2	16
29	3	4	2	1	1	2	3	16
30	1	1	3	2	4	2	3	16
31	3	1	2	2	3	2	3	16
32	1	4	4	1	2	1	2.5	15.5
33	1	1	3	3	2	2	3.5	15.5
34	1	1	3	1	2	2	2.5	12.5

CPF = Colony Performance Factor

Appendix 2: Publication on "Effect of larval age and supplemental feeding on morphometrics and oviposition in honeybee Apis mellifera scutellata queens".



Journal of Apicultural Research

ISSN: 0021-8839 (Print) 2078-6913 (Online) Journal homepage: http://www.tandfonline.com/loi/tjar20

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To cite this article: Loise Kawira Njeru, Suresh Kumar Raina, Helen Lydiah Kutima, Daisy Salifu, David Tembong Cham, James Ng'ang'a Kimani & Elliud Maundu Muli (2017) Effect of larval age and supplemental feeding on morphometrics and oviposition in honey bee Apis mellifera scutellata queens, Journal of Apicultural Research, 56:3, 183-189, DOI: 10.1080/00218839.2017.1307714

To link to this article: https://doi.org/10.1080/00218839.2017.1307714

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Journal of Apicultural Research, 2017
Vol. 56, No. 3, 183–189, http://dx.doi.org/10.1080/00218839.2017.1307714

ORIGINAL RESEARCH ARTICLE

Effect of larval age and supplemental feeding on morphometrics and oviposition in honey bee *Apis mellifera scutellata* queens

Loise Kawira Njeru[a,b]*, Suresh Kumar Raina[a], Helen Lydiah Kutima[b], Daisy Salifu[a], David Tembong Cham[a], James Ng'ang'a Kimani[a] and Elliud Maundu Muli[a,c]

[a]*International Centre of Insect Physiology and Ecology (icipe), Nairobi, Kenya;* [b]*Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya;* [c]*South Eastern Kenya University (SEKU), Kitui, Kenya*

(*Received 31 May 2016; accepted 9 March 2017*)

Although the effects of numerous factors (such as age of grafted larvae, supplemental feeding and mating) have been studied on the honey bees of Europe and America, they remain unknown for African bee races. To bridge this gap, a study was undertaken at the *icipe* Karura forest apiaries in Kenya to determine the effect of larval age and supplemental feeding on morphometrics and oviposition in the honey bee *Apis mellifera scutellata* queens. Queens were reared in 12 colonies with two feeding regimes, fed and not fed. Five larval age groups: 6, 12, 24, 36, and 48 h old, were grafted from each colony. We measured the fresh weight, spermatheca volume and the external parameters of the emerged queens and compared the oviposition rate by counting the number of eggs laid daily in naturally mated queens (NM) and artificially inseminated queens (AI). Our results show that age of grafted larvae and supplemental feeding significantly affect the morphometrics of the reared queens ($p = 0.001$), while oviposition rate is more or less the same in NM and AI. This work could eventually be used to select the best breed of honey bee subspecies in East Africa and improve queen rearing methods.

Efecto de la edad larvaria y la alimentación suplementaria sobre la morfometría y la ovoposición en reinas de la abeja de la miel *Apis mellifera scutellata*

Aunque se han estudiado los efectos de numerosos factores (como la edad de larvas introducidas, la alimentación suplementaria y el apareamiento) en las abejas de la miel de Europa y América, en las razas de abejas africanas siguen siendo desconocidos. Para solventar esto, se ha llevado a cabo un estudio los apiarios *icipe* del bosque de Karura en Kenia para determinar los efectos de la edad larvaria y la alimentación suplementaria sobre la morfometría y la ovoposición en reinas de la abeja de la miel *Apis mellifera scutellata*. Las reinas se criaron en 12 colmenas con dos regímenes de alimentación, alimentadas y no alimentadas. Cinco grupos de edad larvarios: de 6, 12, 24, 36 y 48 horas de edad, se introdujeron en cada colmena. Medimos el peso en fresco, el volumen de la espermateca y los parámetros externos de emergencia de las reinas y comparamos la tasa de ovoposición contando el número de huevos puestos diariamente en reinas apareadas naturalmente (AM) y en reinas inseminadas artificialmente (IA). Nuestros resultados muestran que la edad de las larvas introducidas y la alimentación suplementaria afectan significativamente a la morfometría de las reinas criadas (p = 0,001), mientras que la tasa de ovoposición es más o menos la misma en AM e IA. Este trabajo podría usarse para seleccionar las mejores variedades de las subespecies de las abejas de la miel de África Oriental y mejorar los métodos de cría de las reinas

Keywords: *Apis mellifera scutellata*; queen rearing; artificial insemination; natural mating; oviposition rate; Kenya; East Africa

Introduction

Selective breeding in the natural environment or controlled mating of virgin honey bee queens using artificial insemination techniques can be used to improve the quality of different honey bee stocks (Cobey, 2007; Pérez-Sato, Châline, Martin, Hughes, & Ratnieks, 2009). While instrumentally inseminated honey bee queens initiate oviposition much later than naturally mated queens (NM) (Woyke et al., 2008), their performance is comparable to that of NMs (Oxley, Hinhumpatch, Gloag, & Oldroyd, 2010). By selectively raising their own queens, beekeepers can take control of the characteristics they desire in their bee stock (Cobey, Tarpy, & Woyke, 2013).

Producing quality queens can improve the performance of honey bee colonies. This quality, however, depends on a number of physical characteristics (such as spermatheca size, live weight, number of ovarioles, and number of spermatozoa in the spermatheca) (Delaney, Keller, Caren, & Tarpy, 2011; Hatjina et al., 2014). Numerous factors can affect these physical characteristics, with age of the grafted larvae being an important factor (Mahbobi, Farshineh-Adl, Woyke, & Abbasi, 2012). Tarpy, Hatch, and Fletcher (2000) found that the reproductive potential/quality of queens reared from younger larvae could be higher. Also, providing a supplemental diet to the rearing colony has been shown

*Corresponding author. Email: lkawira@icipe.org

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to affect the quality of grafted larvae, and thus queen quality. A quality queen should have high live weight and number of ovarioles, large size spermatheca (with high number of spermatozoa), and be disease and pest-free (Hatjina et al., 2014). In queen rearing, pollen, nectar and syrup are important nutritional factors (Estegamat & Gholami, 2010). Supplemental feeding significantly increases most morphological characteristics of the reared queens (Mahbobi et al., 2012).

In Europe, the effect of factors such as age of grafted larvae and supplemental feeding on queen quality has been studied and reported to vary across different honey bee races (Hatjina et al., 2014). According to Mahbobi et al. (2012) queen bees reared from 1-day-old larvae are significantly heavier at emergence and have significantly larger spermatheca than queens reared from 2- and 3-day-old larvae.

Information on the effect of age of grafted larvae and supplemental feeding on queen quality is lacking in *A. m. scutellata,* which is one of the most widely distributed East African honey bee races. Little is known about the benefits of queen rearing and the implications of rearing queens from different larval ages in Kenya. This study was, therefore, carried out to: (a) determine the effect of the age of grafted larvae and supplemental feeding on *A. m scutellata* honey bee queen physical parameters, and (b) compare oviposition trends in artificially inseminated queens (AI) and NM.

Materials and methods

Study site

The study was conducted at Karura forest in Kenya at *icipe* Apiary 1 (36.8347°E–1.23442°S) and *icipe* Apiary 2 (36.81416°E–1.24525°S) during the months of November 2014 to May 2015. The rainfall ranges from 930 mm to 1250 mm while temperature ranges between 8 and 28 °C. The dominant vegetation cover around the apiaries is *Eucalyptus saligna* (Myrtaceae) and *Croton megalocarpus* (Euphorbiaceae).

Breeder colony selection

Thirty-four colonies of *A. m. scutellata* were evaluated for their performance for selection of four breeder colonies using honey production, varroa mite load, hygienic behavior, colony strength, swarming behavior, and maximum brood surface area (Gregorc & Lokar, 2010; Spivak & Reuter, 1998). We measured the above-mentioned colony phenotypes to select outstanding breeder colonies from the population rather than randomly choosing the colonies, which could result in undesirable physiognomies. Liquid nitrogen was used to freeze the brood area. The frames containing the frozen brood were removed after 24, 48 and 72 h and the number of uncapped cells recorded. A five-point scale was used to score for hygienic behavior 5 (>95%), 4 (90–95%), 3 (80–89%), 2 (70–79%), 1 (<70%), (Spivak & Reuter, 1998). The sugar-shake method was used to estimate varroa mite load (Ellis & Macedo, 2001). The infestation level was recorded using the ratings, high, moderate, and low, and the percentage mite infestation was calculated by dividing the number of collected mites by the number of bees sampled multiplied by 100 (Dietemann et al., 2013).

Queen rearing with different larval age groups

Five larval age groups, namely 48, 36, 24, 12 and 6 h-old larvae were used. A queen from one of the breeder colonies was confined to provide larvae of the right age for grafting starting with 48 h. Dark empty worker combs were supplied to the breeder colony in subsequent manner to suit the 48, 36, 24, 12 and 6-h-old larvae prior to grafting. The confinement procedure started at 18.00 h of day 1 to 22.00 h on the same day, to provide eggs for 48-h-old larvae, and the frame containing the eggs labelled 48 h. On Day 2, the queen was confined from 06.00 to 10.00 h, to provide eggs for 36 h old larvae, and the frame containing the eggs were labelled 36 h. On the same day at 18.00–22.00 h, the queen was confined to provide eggs for 24 h old larvae, and the frame labelled applicably. On Day 3 at 06.00–10.00 h, the queen was confined to provide eggs for 12 h old larvae, and the frame labelled applicably. On the same day, the queen was confined at 24.00–04.00 h to provide eggs for 6 h old larvae, and grafting was done on the sixth day starting from Day 1 of confinement at 10.00 h. Queens were reared using the Doolittle (1915) grafting method in 12 honey bee colonies of equivalent strength having 10 frames of bees mothered by one-year-old queens of *A. m. scutellata*. The colonies were randomly assigned to two feeding regimens; fed and unfed. Pollen supplement (icing sugar, milk powder, and honey in the ratio of 2:2:1, respectively) was given four weeks before grafting, which continued until the queen cells were capped. Twenty-five wax cups were prepared and fixed on each grafting frame with two grafting bars. Twelve selected cell builder colonies with equal worker population headed by a one-year-old queen were used to raise the queens. The colonies were rendered queenless for 24 h before grafting. Labelled frames with larvae of different ages were then taken out and grafting done using a grafting tool. This grafting was done concurrently from different frames containing different larval age groups. Each larvae age set was grafted into 5 labelled cells per grafting frame, and acceptance rate recorded. One day before the estimated time of emergence of the virgin queens, capped queen cells in each experimental colony were confined using hair rollers and queen banks, to prevent the queens from attacking each other after emergence.

Morphometric analysis of the external and internal body characteristics of newly emerged queens

The newly-emerged queens were first immobilized at −20°C for about 3 min, to record the wet weight using a digital scale to the nearest 0.1 mg, then immediately killed and the following morphometrics measured: (1) external body characteristics, namely head width, head length, thorax width, thorax length, right wing width, wing length; and (2) internal body characteristics, namely spermatheca length and width. A Stemi 2000-C dissecting microscope with a Zeiss camera (Axiocam 105 color) (Carl Zeiss Microscopy GmbH 37081 GÖttingen, Germany) mounted on a computer and a monitor were used to measure the body parameters. The magnification used was lens 1.6×, camera 0.5× and zoom 0.65× = total magnification 0.52×. A pair of fine forceps was used to separate each individual part for measurement. To access the spermatheca, the queens' abdomens were dissected using a scalpel and pair of forceps. The measured length and width of spermatheca was used to calculate spermatheca volume (SV) according to the formula: SV = $(4/3)(\pi)(r^3)$, where r is the average of length and width of spermatheca (Hatch, Tarpy, & Fletcher, 1999). Spermatheca volume is an indicator of storage capacity of semen.

Evaluating the oviposition rate in naturally mated and artificially inseminated queens

Drone rearing was carried out according to Rhodes (2002). In the selected breeder colony, the queen was confined in a confinement cage fitted with an empty drone comb, and released immediately after eggs were laid on the comb. Immediately after capping, the drone brood was moved into the super above the queen excluder. Once the drones emerged, the super was opened only early in the morning to control drifting.

Rearing of queens for artificial insemination and natural mating

Queens were reared from one of the breeder colonies using larvae ages capable of producing best quality queens (12–24 h old larvae). By dividing selected colonies and then introducing developing queen cells from the breeder colony on the 10th day of larvae grafting, nucleus colonies were raised. A developing queen cell was introduced into each of the 24 nucleus colonies. Twelve of the 24 nucleus colonies containing introduced queen cells had their entrances confined using queen excluders, to restrict the queens from undertaking nuptial flights. This group of queens was used for instrumental insemination. The remaining 12 nucleus colonies had open entrances that allowed the virgin queens to mate naturally during nuptial flights.

Artificial insemination procedure

Artificial insemination was carried out using the methods described by Collins (2000) and Cobey et al. (2013). A Swienty insemination apparatus was used together with a Stemi 2000-C dissecting microscope (Carl Zeiss Microscopy GmbH 37081 GÖttingen, Germany) to carry out the insemination procedure. On the 6th day after emergence, virgin queens were given preparatory carbon dioxide treatment for 7 min. They were then returned to their respective colonies awaiting insemination on the following day. Each queen was inseminated with 8 μl of semen, returned to the queen cage and given approximately 10 min to revive. The queens were then re-introduced into their respective colonies using candy (prepared by mixing honey and icing sugar). The queens were monitored every 24 h, to determine the first egg-laying date and the number of eggs laid by providing empty combs to the queens. Egg counting was done on a daily basis for 14 days until both inseminated and NMs increased their egg numbers, then biweekly and monthly using a transparent sheet of paper for both inseminated and NMs. A comb containing newly laid eggs was taken out and laid horizontally on a flat surface. A transparent sheet was placed on top, and using a marker pen, the cells with eggs were circled and then counted later; one sheet for side A and another for side B of a single comb, then the total was calculated. The queen was then given another empty comb (or if there was more space in the previous comb, the areas with eggs were marked with a marker pen and the comb left for the queen to lay again).

Data analysis

The proportion data on a queen's acceptance were analyzed using generalized linear model (glm) with logit link and binomial distribution error to evaluate the effect of the factors larvae age and feeding group. Effect of factors for a glm is reflected in the deviance that has an approximate chi-square distribution; hence, the chi-square values are presented as test statistics. Means were separated using adjusted Tukey, implemented using the *glht function* of the multcomp package (Hothorn, Bretz, & Westfall, 2008). Multivariate analysis of variance (MANOVA) was used, to evaluate the effect of supplemental feeding on queen quality (fed and unfed groups of colonies) for all nine measured morphometrics simultaneously on each of the four larvae age groups (36, 24, 12 and 6 h). Univariate analysis (one-way ANOVA) followed by SNK *post hoc* test were used to further compare means of the individual morphometrics within each group where MANOVA (λ) was significant.

Results

Acceptance of grafted queens

Results on the analysis of proportion data on queen acceptance showed that age and supplemental feeding

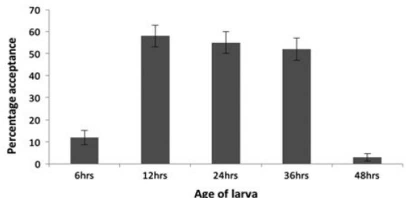

Figure 1. Grafted larvae acceptance rate expressed as percentage larvae grafted for the various larvae age groups (averaged over fed and unfed colonies).

affected *A. m. scutellata* queen acceptance rate independently (χ^2 = 0.35, *df* = 4, *p* = 0.99); however, the main effect of supplemental feeding was significant (χ^2 = 5.01, *df* = 1, *p* = 0.025). Colonies fed with supplemental diet had higher acceptance rate (41%) compared to unfed colonies (31%). The main effect of larval age was also significant (χ^2 = 142.53, *df* = 4, $p < 0.0001$). Larvae at 6 and 48 h had lower acceptance rate as compared to age groups 12, 24, and 36 h (Figure 1).

Morphometric variability of queens reared from various larval ages in fed and unfed colonies

MANOVA suggested significant interaction between larval age and supplemental feeding on the morphometrics of *A. m. scutellata* queens; Wilks' λ = 0.29, $p < 0.0001$. The difference in ages of the grafted larvae within the fed group of experimental colonies significantly affected the queen characteristics (Table 1). The 48-h-old larvae were eliminated from the analysis due to poor acceptance. The SNK *post hoc* test revealed that queens reared from 12- and 24-h-old larvae were on average heavier and larger in most morphometric measurements than those reared from larval ages 6 and 36. The only constant parameter across groups was the thorax width (Table 1).

Comparison of morphometric parameters of queens reared from different larvae ages within the unfed groups of colonies produced different results. Queens grafted from the different larvae ages had almost similar external morphometric measurements (Table 2). The internal morphometric parameters (spermatheca width and length) varied in queens reared from the different larvae age groups. Larvae ages 12 and 24 produced queens with larger spermatheca compared to the rest.

Comparison of measured parameters of queens grafted from the two groups of fed and unfed colonies revealed that colony feeding affected almost all the external morphometrics of the reared queens apart from the wing length and width (Table 3). The spermatheca volume was significantly larger in queens reared from fed than those from unfed colonies.

Oviposition rates in naturally mated and artificially inseminated queens

Queens that were allowed to go on nuptial flight (naturally-mated queens) started laying eggs earlier (on the 6th day after emergence) relative to the instrumentally inseminated queens (on the 11th day after emergence) (Figure 2). In addition, NMs laid slightly more eggs on the first egg-laying day compared to instrumentally inseminated queens. On the second day of egg-laying, oviposition was levelled up for both inseminated- and naturally-mated queens (Figure 2).

Discussion

The higher acceptance rate obtained for larvae ages 12, 24 and 36 h within both fed and unfed groups indicates that these larvae ages are suitable for grafting *A. m. scutellata* queens irrespective of feeding scheme. The low acceptance rate of the 48 h old larvae was likely due to their size and the injuries sustained during grafting, as a result of repeated attempts to scoop the large larva. This finding is consistent with reports by Muli, Raina, and Mueke (2005) on the acceptance rate of *A. m. scutellata* and *A. m. monticola* larvae, but inconsistent with the acceptance rates reported for *A. m. anatoliaca* (Gencer, Shah, & Firatli, 2000). The 6 h old larvae were

Table 1. Mean ± SE of measured morphometrics of queen honey bees grafted from different larvae ages from fed colonies.

	Larval age in hours			
Parameters	6	12	24	36
Wet weight (mg)	0.17 ± 0.020[b]	0.16 ± 0.020[b]	0.18 ± 0.009[a]	0.14 ± 0.018[c]
Head width (mm)	1.78 ± 0.154[b]	1.87 ± 0.065[a]	1.88 ± 0.065[a]	1.86 ± 0.076[a]
Head length (mm)	1.84 ± 0.185[b]	1.81 ± 0.210[b]	1.97 ± 0.062[a]	1.84 ± 0.117[b]
Thorax width (mm)	2.33 ± 0.016[a]	2.30 ± 0.220[a]	2.30 ± 0.151[a]	2.30 ± 0.151[a]
Thorax length (mm)	2.82 ± 0.015[ab]	2.71 ± 0.214[b]	2.88 ± 0.106[a]	2.75 ± 0.209[ab]
Wing width (mm)	1.65 ± 0.010[a]	1.61 ± 0.062[a]	1.51 ± 0.088[b]	1.62 ± 0.108[a]
Wing length (mm)	5.02 ± 0.232[a]	4.86 ± 0.276[ab]	4.70 ± 0.117[b]	4.77 ± 0.280[b]
Spermatheca volume (mm^3)	0.74 ± 0.054[b]	1.02 ± 0.033[a]	0.94 ± 0.023[a]	0.94 ± 0.024[a]

Notes: Mean separation is based on univariate analysis of variance. Means followed by the same letter within a row are not significantly different (Student–Neuman–Keuls test, α = 0.05; n = 102).

Table 2. Mean ± SE of measured morphometrics of queens grafted from different larvae ages from unfed colonies.

Parameters	Larval age in hours			
	6	12	24	36
Wet weight (mg)	0.15 ± 0.008[a]	0.14 ± 0.019[a]	0.16 ± 0.032[a]	0.16 ± 0.005[a]
Head width (mm)	1.72 ± 0.146[b]	1.85 ± 0.075[a]	1.80 ± 0.054[a]	1.85 ± 0.056[a]
Head length (mm)	1.79 ± 0.185[a]	1.77 ± 0.215[a]	1.88 ± 0.105[a]	1.80 ± 0.229[a]
Thorax width (mm)	2.33 ± 0.018[a]	2.28 ± 0.269[a]	2.19 ± 0.217[a]	1.93 ± 0.067[b]
Thorax length (mm)	2.82 ± 0.016[a]	2.64 ± 0.230[a]	2.68 ± 0.213[a]	2.39 ± 0.283[b]
Wing width (mm)	1.64 ± 0.013[a]	1.57 ± 0.072[a]	1.58 ± 0.113[a]	1.65 ± 0.056[a]
Wing length (mm)	4.97 ± 0.251[a]	4.82 ± 0.218[a]	4.79 ± 0.190[a]	4.91 ± 0.069[a]
Spermatheca volume (mm^3)	0.68 ± 0.071c	0.91 ± 0.037a	0.85 ± 0.026a	0.36 ± 0.013b

Notes: Mean separation is based on univariate analysis of variance. Means followed by the same letter within a row are not significantly different (Student–Neuman–Keuls test, $\alpha = 0.05$; $n = 78$).

Table 3. Comparison of measured parameters of queens produced from fed and unfed groups of colonies.

Parameter	Mean ± SE of fed queens (n = 102)	Mean ± SE of unfed queens (n = 78)	t-value	p-value
Wet weight	0.163 ± 0.002	0.154 ± 0.003	2.68	0.008
Head width	1.862 ± 0.008	1.828 ± 0.009	2.87	0.005
Head length	1.873 ± 0.016	1.812 ± 0.022	2.26	0.025
Thorax width	2.264 ± 0.002	2.151 ± 0.029	3.29	0.001
Thorax length	2.779 ± 0.023	2.591 ± 0.032	5.47	<0.0001
Wing width	1.585 ± 0.010	1.601 ± 0.010	1.11	0.270
Wing length	4.795 ± 0.025	4.843 ± 0.021	1.40	0.162
Spermatheca volume (mm^3)	0.950 ± 0.017	0.717 ± 0.031	7.10	<0.0001

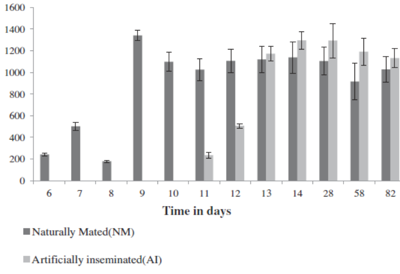

Figure 2. Oviposition pattern in naturally-mated (NM) and artificially-inseminated (AI) *Apis mellifera scutellata* queens.

tiny and delicate and had little royal jelly supplied to them at the time of grafting; hence, their low acceptance. The weight of queens grafted from all the accepted larval ages (6, 12, 24, and 36 h old) indicated that all the colonies were productive and of equal strength (Delaney et al., 2011; De Souza, Bezzera-Laure, Francoy, & Gonçalves, 2013; Kahya, Gençer, & Woyke, 2008). However, within the fed group, the 24 h old larvae had heavier queens. The quality of a queen and thus the colony, depends on the spermatheca size and volume (Carreck et al., 2013). Larger spermatheca volume of queens reared from the 12 and 24 h old larvae from within the fed and unfed colonies, therefore, suggests that these two age groups are best for rearing *A. m. scutellata* queens, which is in agreement with the findings of Büchler et al. (2013). Even though the 6 and 48 h old larvae produced queens with high fresh weights, their low acceptance and spermatheca size makes them less suitable for grafting with regard to queen rearing.

Our findings demonstrate that supplemental feeding affects most morphometric characters of the reared queens and agree with those reported by Mahbobi et al. (2012) for the Iranian honey bee *A. m. meda* queens. The interaction between age and feeding implies that supplemental feeding may affect the weight of the resultant queens of some larvae ages more than others.

The onset of oviposition in NMs commenced earlier than the instrumentally inseminated queens. These results confirm previous reports that indicated that instrumentally inseminated honey bee queens initiate oviposition much later than NMs (Woyke et al., 2008). Our observation that there is no significant difference in the number of eggs laid by AI and NM is supported by a study that has demonstrated that instrumentally inseminated queen bees perform equally well as NM bees (Oxley et al., 2010). Nevertheless, given that there is no selection of traits in NM as opposed to AI queens, artificial insemination remains the best method to control mating and selection of desired traits.

Age of the grafted larvae affects the quality of the queens produced in *A.m. scutellata* in terms of spermatheca volume, which is an important parameter in determining the quality of a queen bee. This study shows that the appropriate age for grafting *A. m. scutellata* larvae is

12- to 24-h. Supplemental feeding is another important factor that can improve the production of high quality *A. m. scutellata* queens as indicated by our findings. This study provides evidence that artificially inseminated *A. m. scutellata* queens perform equally well compared to NMs. Artificial insemination technique is important in breeding programs, and the results can be used for research on African *A. mellifera* subspecies. However, further study is required to establish any correlations between the quality of the queens produced from the different age groups and colony health or overall colony productivity.

Acknowledgements

The authors would like to thank the Bee Health Project of the International Center of Insect Physiology and Ecology (*icipe*) for providing the research facilities for this study. We also express our gratitude to the entire Bee Health staff for their support.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This project was funded by the European Union (EU) under grant [number EU/ICIPE 2013/313–659] and the International Fund for Agricultural Development (IFAD) under grant [number IFAD/ICIPE 1229]. We also gratefully acknowledge the financial support for this research by the following organizations and agencies: UK Aid; Swedish International Development Cooperation Agency (Sida); the Swiss Agency for Development and Cooperation (SDC); and the Kenyan Government. The views expressed herein do not necessarily reflect the official opinion of the donors.

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