

**EVALUATION AND MOLECULAR CHARACTERIZATION OF
HYGIENIC TRAITS IN HONEY BEE (*Apis mellifera* Linnaeus)**

Dissertation

**Submitted to the Punjab Agricultural University
in partial fulfilment of the requirements
for the degree of**

**DOCTOR OF PHILOSOPHY
in
ENTOMOLOGY
(Minor Subject: Biotechnology)**

By

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(L-2013-A-13-D)**

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CERTIFICATE I

This is to certify that the dissertation entitled, '**Evaluation and molecular characterization of hygienic traits in honey bee (*Apis mellifera* Linnaeus)**' submitted for the degree of **Ph.D.** in the subject of **Entomology** (Minor subject: **Biotechnology**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Bharathi Mohindru (Admission No. L-2013-A-13-D)** under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE II

This is to certify that the dissertation entitled, '**Evaluation and molecular characterization of hygienic traits in honey bee (*Apis mellifera* Linnaeus)**' submitted by **Bharathi Mohindru (Admission No. L-2013-A-13-D)** to the Punjab Agricultural University, Ludhiana, in partial fulfilment of the requirements for the degree of **Ph.D.** in the subject of **Entomology** (Minor subject: **Biotechnology**) has been approved by the Student's Advisory Committee after an oral examination on the same in collaboration with an External Examiner.

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ABSTRACT

Studies on 'Evaluation and molecular characterization of hygienic traits in honey bee (*Apis mellifera* Linnaeus)' were conducted at PAU, Ludhiana (India), during 2015-2017. The studies included screening of 100 *A. mellifera* colonies for comparative hygienic behaviour response, molecular characterization through genome wide and candidate gene based markers for hygienic behaviour, and validation of the selected hygienic colonies against *Varroa destructor* infestation. Out of the 100 colonies screened, 45 were hygienic, and 55 were non-hygienic out of which 42 were intermediary (71-80% removal of pricked brood) and 13 were the least hygienic (<71% emptying of pricked brood). After 24 h of brood pricking, mean brood removal was 83.65 per cent (range: 80.22-91.0 %) among 45 colonies while it was 73.05 per cent (range: 46.78-79.56%) in the remaining 55 colonies. In the seven selected most hygienic colonies, after 24 h, mean of 91.40 per cent brood was removed (range: 87.22-94.89%), while it was 47.63 per cent (range: 43.56-52.44%) in the three most non-hygienic selected colonies. The hygienic colonies took 20.6 h to express the hygienic trait while the non-hygienic colonies took 44.6 h. After 24 h of *V. destructor* inoculation into the brood in the hygienic colonies, 93.43 and 95.23 per cent mean of brood removal was recorded during autumn and spring, while in the non-hygienic colonies, it was only 61.90 and 77.24 per cent, respectively, to achieve cent per cent cleaning of cells, the hygienic colonies took a mean of 28 and 25.71 h, while non-hygienic colonies took 50.67 and 47.33 h, during autumn and spring, respectively. The five SSR markers, reported to be linked with hygienic behaviour, showed polymorphism, and did not differentiate the colonies among hygienic and non-hygienic groups. Out of the 40 SSR markers used to study the genetic diversity between the hygienic and non-hygienic colonies, 37 markers were polymorphic; the diversity index value ranged between 0.37-0.92, marker index value between 0.24-6.41 and the PIC value between 0.23-0.77. The clustering pattern between the hygienic and non-hygienic colonies for each of the three brood cycles produced by neighbour-joining dendrogram showed that the ten colonies (7 most hygienic and 3 most non-hygienic) divided into three clusters. The clustering pattern depicting all the three brood cycles together, also consisted of three clusters; cluster I with four hygienic colonies, cluster II with 3 hygienic and 2 non-hygienic colonies and cluster III consisted of one non-hygienic colony. The grouping pattern of all the colonies varied in all the three brood cycles except for two hygienic colonies (6H and 7H) which consistently formed one group. The newly designed 34 gene specific primers amplified all 300 genomic DNA samples from both the types of colonies with no length polymorphism observed between the hygienic and non-hygienic individuals. Sequencing of the three candidate genes revealed no variation, implying these as totally conserved genes. One gene *GB19509* showed six differential SNPs between hygienic and non-hygienic colonies.

Keywords: *Apis mellifera*, candidate genes, defense mechanism, honey bee, hygienic behaviour, molecular characterization, pin killed brood method, SSR markers, *Varroa destructor*

Signature of Major Advisor

Signature of the Student

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ਨਿਰਦੇਸ਼

ਮੌਜੂਦਾ ਅਧਿਐਨ 'ਸ਼ਹਿਦ ਦੀ ਮੱਖੀ (ਏਪਿਸ ਮੈਲੀਫੇਰਾ ਲੀਨੀਅਸ) ਵਿੱਚ ਸਵੱਛਤਾ ਦੇ ਗੁਣਾਂ ਦਾ ਆਣਵਿਕ ਚਿੱਤਰ-ਚਿੱਤਰਨ ਅਤੇ ਮੁਲਾਂਕਣ' ਸਿਰਲੇਖ ਅਧੀਨ ਸੰਨ 2015-17 ਦੌਰਾਨ ਪੀ.ਏ.ਯੂ. ਲੁਧਿਆਣਾ (ਭਾਰਤ) ਵਿਖੇ ਕੀਤਾ ਗਿਆ। ਅਧਿਐਨ ਦੌਰਾਨ ਤੁਲਨਾਤਮਕ ਸਵੱਛ ਪ੍ਰਤੀਕਿਰਿਆ ਲਈ, ਸਵੱਛ ਵਤੀਰੇ ਲਈ ਜੀਨੋਮ ਵਿਸ਼ਲੇਸ਼ਣ ਅਤੇ ਪ੍ਰਤੀਆਸ਼ੀ ਜੀਨ ਅਧਾਰਤ ਮਾਰਕਰਾਂ ਰਾਹੀਂ ਆਣਵਿਕ ਚਿੱਤਰ-ਚਿੱਤਰਨ ਅਤੇ ਵਰੋਆ ਡਿਸਟਕਟਰ ਚਿਚੜੀ ਦੇ ਲਾਗ ਵਿਰੁੱਧ ਚੋਣਵੇਂ ਸਵੱਛ ਕਟੁੰਬਾਂ ਦੀ ਪ੍ਰਮਾਣਿਕਤਾ ਦਾ ਮੁਲਾਂਕਣ ਕਰਨ ਲਈ ਏਪਿਸ ਮੈਲੀਫੇਰਾ ਦੇ 100 ਕਟੁੰਬਾਂ ਦੀ ਜਾਂਚ ਕੀਤੀ ਗਈ। ਜਾਂਚੇ ਗਏ 100 ਕਟੁੰਬਾਂ ਵਿੱਚੋਂ 45 ਸਵੱਛਤਾ ਭਰਪੂਰ, ਅਤੇ 55 ਘੱਟ-ਸਵੱਛ ਸਨ ਜਿਹਨਾਂ ਵਿੱਚੋਂ 42 ਕਟੁੰਬ ਦਰਮਿਆਨੇ (71-80%) ਅਤੇ 13 ਕਟੁੰਬ ਸਭ ਤੋਂ ਘੱਟ ਸਵੱਛਤਾ ਵਾਲੇ (<71%) ਪਾਏ ਗਏ। 24 ਘੰਟੇ ਮਗਰੋਂ, 45 ਕਟੁੰਬਾਂ ਵਿੱਚੋਂ ਮਰੇ ਹੋਏ ਬਰੂਡ ਕੱਢੇ ਜਾਣ ਦੀ ਔਸਤ 83.65 ਪ੍ਰਤੀਸ਼ਤ (80.22-91.0%) ਸੀ ਅਤੇ 55 ਕਟੁੰਬਾਂ ਵਿੱਚ 73.05 ਪ੍ਰਤੀਸ਼ਤ (46.78-79.56%) ਸੀ। 24 ਘੰਟੇ ਮਗਰੋਂ, ਚੁਣੇ ਗਏ ਸਭ ਤੋਂ ਵਧੇਰੇ ਸਵੱਛਤਾ ਵਾਲੇ ਸੱਤ ਕਟੁੰਬਾਂ ਵਿੱਚ, ਮਰੇ ਹੋਏ ਬਰੂਡ ਦੀ ਕੱਢੇ ਜਾਣ ਦੀ ਔਸਤ 91.40 ਪ੍ਰਤੀਸ਼ਤ (87.22-94.89%) ਦਰਜ ਕੀਤੀ ਗਈ, ਜਦੋਂਕਿ ਸਭ ਤੋਂ ਘੱਟ ਸਵੱਛ ਤਿੰਨ ਕਟੁੰਬਾਂ ਵਿੱਚ ਇਹ 47.63 ਪ੍ਰਤੀਸ਼ਤ (43.56-52.44%) ਸੀ। ਸਵੱਛਤਾ ਭਰਪੂਰ ਕਟੁੰਬਾਂ ਨੇ ਸਵੱਛਤਾ ਦੇ ਗੁਣਾਂ ਨੂੰ ਦਰਸਾਉਣ ਲਈ ਔਸਤਨ 20.6 ਘੰਟੇ ਦਾ ਸਮਾਂ ਲਿਆ ਜਦੋਂਕਿ ਘੱਟ ਸਵੱਛਤਾ ਵਾਲੇ ਕਟੁੰਬਾਂ ਨੇ ਇਸ ਪੱਧਰ ਤੱਕ ਪੁੱਜਣ ਲਈ ਔਸਤਨ 44.6 ਘੰਟੇ ਦਾ ਸਮਾਂ ਲਿਆ। ਪਤਝੜ ਅਤੇ ਬਹਾਰ ਰੁੱਤ ਦੌਰਾਨ ਚੁਣੇ ਗਏ ਸਵੱਛ ਕਟੁੰਬਾਂ ਦੇ ਬਰੂਡ ਵਿੱਚ ਵਰੋਆ ਡਿਸਟਕਟਰ ਦੀ ਲਾਗ ਲਗਾਉਣ ਤੋਂ 24 ਘੰਟੇ ਮਗਰੋਂ, ਕ੍ਰਮਵਾਰ ਔਸਤਨ 93.43 ਅਤੇ 95.23 ਪ੍ਰਤੀਸ਼ਤ ਲਾਗ ਵਾਲਾ ਬਰੂਡ ਕੱਢਿਆ ਦਰਜ ਕੀਤਾ ਗਿਆ, ਜਦੋਂਕਿ ਤਿੰਨ ਘੱਟ ਸਵੱਛ ਕਟੁੰਬਾਂ ਵਿੱਚ, ਇਹ ਦਰ ਕ੍ਰਮਵਾਰ ਸਿਰਫ 61.90 ਅਤੇ 77.24 ਪ੍ਰਤੀਸ਼ਤ ਦਰਜ ਕੀਤੀ ਗਈ; ਸੈਲਾਂ ਦੀ ਸੋਫੀਸਦੀ ਸਫਾਈ ਲਈ, ਪਤਝੜ ਅਤੇ ਬਹਾਰ ਰੁੱਤ ਦੌਰਾਨ, ਸਵੱਛ ਕਟੁੰਬਾਂ ਨੇ ਕ੍ਰਮਵਾਰ ਔਸਤਨ 28 ਅਤੇ 25.71 ਘੰਟੇ ਦਾ ਸਮਾਂ ਲਿਆ, ਜਦੋਂਕਿ ਘੱਟ ਸਵੱਛ ਕਟੁੰਬਾਂ ਨੇ ਔਸਤਨ 50.67 ਅਤੇ 47.33 ਘੰਟੇ ਦਾ ਸਮਾਂ ਲਿਆ। ਸਵੱਛ ਵਤੀਰੇ ਨਾਲ ਸਬੰਧਤ ਪੰਜ ਐਸ.ਐਸ.ਆਰ. ਮਾਰਕਰਾਂ ਨੇ ਜ਼ਿਆਦਾ ਸਵੱਛ ਅਤੇ ਘੱਟ ਸਵੱਛ ਸਮੂਹਾਂ ਦਰਮਿਆਨ ਕਟੁੰਬਾਂ ਵਿੱਚ ਕੋਈ ਵਿਭਿੰਨਤਾ ਨਹੀਂ ਦਰਸਾਈ, ਹਾਲਾਂਕਿ ਸਾਰੇ ਮਾਰਕਰਾਂ ਨੇ ਬਹੁਰੂਪਤਾ ਦਰਸਾਈ। ਜ਼ਿਆਦਾ ਸਵੱਛ ਅਤੇ ਘੱਟ ਸਵੱਛ ਕਟੁੰਬਾਂ ਵਿੱਚ ਅਨੁਵਾਂਸ਼ਿਕੀ ਵਿਭਿੰਨਤਾ ਅਧਿਐਨ ਲਈ ਵਰਤੇ ਗਏ 40 ਐਸ.ਐਸ.ਆਰ. ਮਾਰਕਰਾਂ ਵਿੱਚੋਂ 37 ਮਾਰਕਰ ਬਹੁਰੂਪਕ ਸਨ; ਵਿਭਿੰਨਤਾ ਅੰਕ 0.37-0.92, ਮਾਰਕਰ ਅੰਕ 0.24-6.41 ਅਤੇ ਪੀ.ਆਈ.ਸੀ. ਮਿਕਦਾਰ 0.23-0.77 ਦਰਜ ਕੀਤੀ ਗਈ। ਤਿੰਨਾਂ ਬਰੂਡ ਚੱਕਰਾਂ ਲਈ ਸੱਤ ਜ਼ਿਆਦਾ ਸਵੱਛ ਅਤੇ ਤਿੰਨ ਘੱਟ ਸਵੱਛ ਕਟੁੰਬਾਂ ਵਿੱਚਲੇ ਡੈਂਡਰੋਗ੍ਰਾਮ ਨੂੰ ਤਿੰਨ ਹਿੱਸਿਆਂ ਵਿੱਚ ਸ਼੍ਰੇਣੀਗਤ ਕੀਤਾ ਗਿਆ। ਤਿੰਨ ਬਰੂਡ ਚੱਕਰਾਂ ਨੂੰ ਦਰਸਾਉਣ ਵਾਲੀ ਸ਼੍ਰੇਣੀਗਤ ਸੰਰਚਨਾ ਵਿੱਚ ਸ਼੍ਰੇਣੀ I ਵਿੱਚ ਚਾਰ ਸਰਵੋਤਮ ਸਵੱਛ ਕਟੁੰਬ, ਸ਼੍ਰੇਣੀ II ਵਿੱਚ 3 ਸਰਵੋਤਮ ਸਵੱਛ ਅਤੇ 2 ਘੱਟ ਸਵੱਛ ਕਟੁੰਬ ਅਤੇ ਸ਼੍ਰੇਣੀ III ਵਿੱਚ ਸਿਰਫ 1 ਘੱਟ ਸਵੱਛਤਾ ਵਾਲਾ ਕਟੁੰਬ ਪਾਇਆ ਗਿਆ। ਦੋ ਕਟੁੰਬਾਂ (6H ਅਤੇ 7H) ਤੋਂ ਇਲਾਵਾ ਬਾਕੀ ਦੇ ਸਾਰੇ ਕਟੁੰਬਾਂ ਦੇ ਤਿੰਨਾਂ ਬਰੂਡ ਚੱਕਰਾਂ ਦੀ ਸ਼੍ਰੇਣੀ ਸੰਰਚਨਾ ਵਿੱਚ ਵਿਭਿੰਨਤਾ ਦਰਜ ਕੀਤੀ ਗਈ; ਕਟੁੰਬ 6H ਅਤੇ 7H ਨੇ ਨਿਰੰਤਰ ਤੌਰ ਤੇ ਇੱਕੋ ਸ਼੍ਰੇਣੀ ਬਣਾਈ। 34 ਗੁਣਸੂਤਰਾਂ ਦੇ ਅਧਾਰਿਤ ਪ੍ਰਾਈਮਰਾਂ ਨੇ ਸਰਵੋਤਮ ਸਵੱਛ ਅਤੇ ਘੱਟ ਸਵੱਛ ਐਂਪਲੀਕੋਨਸ ਦਰਮਿਆਨ ਬਿਨਾਂ ਲੰਬਾਈ ਬਹਿਰੂਪਤਾ ਨਾਲ ਸਾਰੇ ਦੇ ਸਾਰੇ 300 ਜੀਨੋਮਿਕ ਡੀ.ਐਨ.ਏ. ਨਮੂਨਿਆਂ ਨੂੰ ਵਿਸ਼ਲੇਸ਼ਣ ਕੀਤਾ। ਤਿੰਨ ਪ੍ਰਤਿਆਸ਼ੀ ਜੀਨਾਂ ਦੇ ਅਨੁਕ੍ਰਮਣ ਤੋਂ ਇਹਨਾਂ ਜੀਨਾਂ ਦੇ ਪੂਰਨ ਤੌਰ ਤੇ ਸੰਰਖਿਆ ਜੀਨ ਹੋਣ ਦਾ ਪਤਾ ਚੱਲਿਆ। ਇੱਕ ਜੀਨ GB19509 ਨੇ ਸਰਵੋਤਮ ਸਵੱਛ ਅਤੇ ਘੱਟ ਸਵੱਛ ਕਟੁੰਬਾਂ ਦਰਮਿਆਨ ਛੇ ਵੱਖੋ-ਵੱਖਰੇ ਐਸ.ਐਨ.ਪੀਜ਼. ਵਿਖਾਏ।

ਮੁੱਖ ਸ਼ਬਦ: ਏਪਿਸ ਮੈਲੀਫੇਰਾ, ਪ੍ਰਤੀਆਸ਼ੀ ਜੀਨ, ਬਚਾਅ ਕਾਰਜ ਵਿਧੀ, ਸ਼ਹਿਦ ਮੱਖੀ, ਸਵੱਛ ਵਿਹਾਰ, ਆਣਵਿਕ ਵਿਸ਼ੇਸ਼ਤਾ, ਪਿਨ ਨਾਲ ਬਰੂਡ ਮਾਰਨ ਦੀ ਵਿਧੀ, ਐਸ.ਐਸ.ਆਰ. ਮਾਰਕਰ, ਵਰੋਆ ਡਿਸਟਕਟਰ

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37.	Mean percentage of brood cells only uncapped in hygienic and non-hygienic colonies at various time intervals after <i>Varroa</i> inoculation during spring 2017
38.	Percentage of brood cells remaining capped in various test colonies at various time intervals after <i>Varroa</i> inoculation during spring 2017
39.	Mean percentage of brood cells remaining capped in hygienic and non-hygienic colonies at various time intervals after <i>Varroa</i> inoculations during spring 2017

CHAPTER I

INTRODUCTION

The honey bee, *Apis mellifera* Linnaeus, originated in eastern tropical Africa, spread to northern Europe and east, into India and the Tien Shan range in China, due to evolutionary forces governed by natural and artificial selection (The Honey Bee Genome Sequencing Consortium 2006). Presently, honey bees are found in all parts of the world except the North Pole and the South Pole.

Honey bees are important not only for the honey with the hive products they yield, but are also the most dominant pollen vectors of agricultural and horticultural crops. One such general estimation suggested that to attain maximum yield, 80 per cent of flowering plants, 90 per cent of all fruit and vegetable crops especially hybrids, and three quarters of staple crops that feed mankind, depend upon pollination by insects (Ombir and Kumar 2010). In general, honey bees enhance agricultural productivity by 20 to 30 per cent (ICIMOD 2004). According to estimation, it has been suggested that one-third of the man's diet can be traced directly or indirectly to pollination by bees (Delaplane and Mayer 2000). Honey bees also play an important ecological role in conserving biodiversity, and thereby, the integrity of global ecosystems (Brown and Paxton 2009).

There were approximately 56 million honey bee colonies in the world during the last decade, and 90 per cent of which were in Europe, Africa and Asia. The world honey production has been estimated at 1.4 million tonnes (Sarswat 2011). China is the largest honey producer contributing 21.20 per cent of the global honey. There were nearly 1.8 million hive honey bee colonies in India (Sarswat 2011, Goyal and Chhuneja 2011) and the total honey production including that from wild honey bees, was estimated at 82,500 tonnes (Anonymous 2016a). The Punjab is the leading state in the country in honey production, accounting for nearly 40 per cent of the nation's apiary honey production (Anonymous 2016b).

In different regions of the world, *A. mellifera* is commonly known as the European, western or the common honey bee and is largely hived for honey, pollen, beeswax, propolis, royal jelly and bee venom (Ratcliffe *et al* 2011). Besides being the major agent for global ecology through pollination (Klein *et al* 2007, Allsopp *et al* 2008), it has been the main model of scientific social behavioral studies (Breed *et al* 2004, Solignac *et al* 2004, Beye *et al* 2006, The Honey Bee Genome Sequencing Consortium 2006).

Biologists are interested in the honey bees' social instincts and behavioural traits (Page and Eber 2002, Crist 2004, The Honey Bee Genome Sequencing Consortium 2006, Shruthi *et al* 2009). They exhibit a wide variety of behavioural phenomena such as kin detection, dance language for communication, regulation of division of labour, a large variety of forms of

learning, resistance to diseases and pests, hygienic behaviour, foraging and defensive behaviours that are of agricultural value. Honey bees influence pollination and hive productivity either directly or indirectly (Hunt *et al* 1998 and 2003, Page *et al* 2000, Arechavaleta-Velasco and Hunt 2003, Oldroyd and Thompson 2007, Paleolog 2009, Shruthi *et al* 2009, Oxley *et al* 2010, Wray *et al* 2011). In beekeeping, behaviour of the whole colony is responsible for most of the attributes of economic importance. The colony is a family comprising of a queen, a few hundred drones (reared and found during breeding season), and worker bees. A queen usually copulates with 10 to 17 drones, thus the colony is a super-family with a common queen mother and sub-families that have different drones as fathers (Rothenbuhler *et al* 1968).

Behavioural traits are complex because the brain, the central mediator of behaviour in most organisms is remarkably complex, coordinating many developmental, physiological and biochemical systems (Page *et al* 1998, Page and Erber 2002, Oldroyd and Thompson 2007). Hence, the evaluation of the social behaviour of the colony is more complicated than that of individuals. These traits are quantitative in nature and are a result of genetic and environment interactions (Page *et al* 2000, Oldroyd and Thompson 2007). The quantitative trait loci (QTLs) in honey bees are important because they display colony-level traits that are a result of the average behaviour of many individual members of the colony (Hunt *et al* 1995, Rueppel *et al* 2004, Bourke 2005, Oldroyd and Thompson, 2007). Colony-level QTLs influencing hygienic behaviour in honey bees have been identified through selective breeding and genetic linkage mapping (Hunt *et al* 1995, Page *et al* 2000, Lapidge *et al* 2002, Oxley *et al* 2010). This means that variation in the genomic regions affects hygienic behaviour in honey bee colonies.

The study of QTLs has a high possibility of determining the genetic architecture of complex traits and identifying candidate genes responsible for hive productivity (Phillips 1999, Page *et al* 2000, Hunt *et al* 1998, 1999 and 2003). Further, the honey bee genome has been sequenced and has thousands of expressed sequence tags (ESTs) and putative genes which need experimental evaluation (Whitfield *et al* 2002). The selected candidate genes can be utilized to genetically improve the honey bee for hive productivity traits. Behaviours such as hygienic (Rothenbuhler 1964a, Moritz 1988, Paleolog 2009), defensive (Hunt *et al* 1998, Lobo *et al* 2003), foraging (Hunt *et al* 1995 and 2007), dance language (von Frisch 1993), stinging (Hunt *et al* 1998), and tracheal mite resistance (Nasr *et al* 2001) have been studied.

Honey bees, because of performing multiple functions, at every age display particular behaviour, thus with time showing a range of behaviour. The change from in-hive tasks such as feeding larvae and comb construction performed by younger bees, to external tasks such as foraging performed by older bees is the most distinguished behavioural change observed in honey bees. Hygienic behaviour is performed by 15 to 20 days old bees which are middle

aged, being older than typical nurse bees but younger than typical foragers (Arathi *et al* 2000).

Honey bees, in general, are exposed to a large diversity of microorganisms when compared with solitary insects, since the colonies of the former offer favourable ambient conditions for many parasites and pathogens, *inter-alia* also owing to a large amount of food and brood reserves like honey, pollen, wax and larvae (Schmid-Hempel 1998). The regulation of temperature and humidity as well as the presence of many closely related and regularly interacting individuals in the colony provide ideal conditions for growth of parasites and microbial pathogens. Infestations and infections negatively affect colony growth and productivity, resulting in economic losses. These include bacteria (*Paenibacillus larvae larvae* (White) Heyndricx *et al*; *Melissococcus plutonius* (White) Truper and De'Clari and *Spiroplasma apis*), protozoa (*Nosema apis* Zander, *Crithidia mellificae* and *Malpighamoeba mellificae* Prell), fungi [*Ascosphaera apis* (Maassen ex Claussel)] and viruses. The macro parasites of honey bees include mites (*Varroa destructor* Anderson and Trueman; *Acarapis woodi* Rennie and *Tropilaelaps clareae* Delfinado and Baker) and the wax moths (*Galleria mellonella* Linnaeus and *Achroia grisella* Fabricius). Among these macro parasites, some additionally act as vectors of viruses and bacteria. Efficient hygienic behaviour increases resistance to various diseases; therefore, keeping bees exhibiting higher degree of hygienic behaviour is recommended as a natural method of minimizing the level of incidence of pests and diseases (Rinderer 1997, Palacio *et al* 2000, Harris 2008, Rinderer *et al* 2010).

Rothenbuhler (1956) was the first to use the term hygienic behaviour which is defined as the ability to identify and remove diseased and parasitized brood from the colony. It is an antiseptic behaviour and differs from grooming [the removal of foreign objects and parasites from oneself (autogrooming) or from another worker honey bee in the nest (allogrooming)]. It was first described in 1930s when researchers sought to determine the mechanism by which some honey bee colonies were resistant to the highly infectious brood disease, American foulbrood, caused by the bacterium *P. larvae* (Park 1937, Park *et al* 1937). Park *et al* (1939) observed that the bees eliminate the presence of disease by immediately removing the diseased larvae. Woodrow and Holst (1942) confirmed that resistance to American foulbrood in the honey bee colony is due to its ability to detect and remove diseased brood before the causal organism, *P. larvae*, reached the infectious spore stage in the diseased larva and thus the early removal of diseased larvae which contained only the non-infectious rods of *P. larvae* prevented spread of disease in the colony, whereas infected brood which contained the highly infectious spores if removed, caused the spread of the disease to other larvae.

Hygienic behaviour consists of two processes; uncapping is the first process which is elimination of the wax cap covering the brood cell performed by some worker honey bees

upon detecting dead or diseased larvae or pupae in the brood comb followed by the removal of the dead brood inside (Spivak and Gilliam 1998a, 1998b). It has emerged as a general system of resistance to brood diseases, and provides significant resistance to a number of economically important pathogens. *A. mellifera*, is the only *Apis* species that does not have a natural parasitic brood mite but is highly susceptible to two mites i.e. *V. destructor* and *T. clareae* that are native to other honey bee species ((Oldroyd 1999). *V. destructor*, is considered the largest threat to apiculture worldwide as it inflicts more damage and higher economic losses than all other known apicultural diseases (Boecking and Genersch 2008). Hygienic and grooming behaviour are two important mite-resistant traits observed in honey bees. It was reported that the honey bees uncap and remove a portion of *V. destructor* mite infested brood (Boecking and Drescher 1992, Spivak 1996). The colonies that express hygienic behaviour are economically important to beekeepers as this behaviour limits the multiplication and spread of infestation (Spivak and Reuter 1998, 2001a, 2001b). In 2006, Ibrahim and Spivak found that hygienic behaviour interrupts the reproduction of mites and the term Varroa Sensitive Hygiene (VSH) was used (Harris 2007). *Varroa* sensitive hygiene (VSH) behaviour involves two major steps: the first step is the uncapping of the infected brood cells and the second step is the removal of the *Varroa* infected pupae from the hive, preventing the mite from reproduction (Harbo and Harris 2005, 2009).

Hygienic behaviour of honey bees benefits the beekeepers with no apparent negative characteristics that accompany the trait. Selection and breeding for hygienic behaviour of honey bees to control the brood diseases and devastating mite pests is preferable as introduction of antibiotics and pesticides into bee colonies results in chemical contamination of bee products such as honey, pollen and wax.

A major component of colony strength is its genetic variability which is a consequence of the multiple mating strategy of the queen. Several studies have reported significant advantages of intracolony genetic diversity which include disease resistance, stable brood thermoregulation, and higher colony growth. Multiple mating generates high genetic similarity among workers within a patriline, and genetic diversity among workers of different patrilines in a honey bee colony. For a trait which is genetically controlled, within a patriline, worker phenotypes should be more similar to each other than to worker phenotypes from different patrilines (Oldroyd and Fewell 2007). In honey bees, such genetically-based variations have been widely reported for different tasks; viz., foraging preferences (Oldroyd *et al* 1992, Oldroyd *et al* 1993) scouting (Dreller 1998), guarding (Giray *et al* 2000), feeding larvae (Chapman *et al* 2007), grooming (Frumhoff and Baker 1988), and dead brood removal (Robinson and Page 1988). Thus, genetic component of honey bees plays an important role in social organization in the colony (Calderone *et al* 1989).

A well established bee breeding programme with the help of marker-assisted selection for the production of high quality, pathogen and parasite resistant stocks, will provide an alternative to chemical usage. Genetic markers that could identify strong hygienic colonies in the field would be very useful tools for selecting choice colonies for breeding and avoiding colonies that do not carry the hygienic marker.

Microsatellite markers have been reported for honey bees (Estoup *et al* 1993, 1994 and 1995; Oldroyd *et al* 1994, Solignac *et al* 2004), and along with mtDNA, these have been used to study genetic structure of honey bee populations (Susnik *et al* 2004, de La Rua *et al* 2006). Microsatellites are tandem repeats of short units of DNA (1-5bp) that occur with high frequency throughout the genomes of many organisms. They are polymorphic in nature and have an advantage of selective neutrality (Estoup *et al* 1993 and 1994; Rowe *et al* 1997, Solignac *et al* 2004).

Genetic distances based on their linkage can be used to answer questions concerning population structure and divergence (Solignac *et al* 2004, de La Rua *et al* 2006, Insuan *et al* 2007). The genetic distance between populations shows how genetically divergent two populations are from each other (Ji *et al* 2011). When the genetic distance is large, the similarity is low and the time they diverge from each other is greater; whilst when the genetic distance is small, the genetic similarity is high and the time they diverge from each other is smaller (Takezaki and Nei 1996, Radloff and Hepburn 1998). Recently, microsatellites have become the marker of choice for ecological and evolutionary studies to answer questions of behavioral ecology, phylogeny, parentage and kinship and have revealed some nuclear introgression from one bee subspecies to another (Franck *et al* 1999, de La Rua *et al* 2003, Insuan *et al* 2007).

The knowledge of genetic relationships and candidate gene identification amongst honey bees is crucial for harnessing their important economic traits for selective breeding, conservation and productivity efforts. Thus, the research under report was proposed to be undertaken to evaluate the existence and the molecular characterization of the hygienic instinct among the honey bee colonies as it is understood from the literature reviewed that it would provide a generalized behavioural response to remove pathogens and parasites from the colony. Thus, the studies were conducted with the objectives of

1. Screening honey bee colonies for hygienic behaviour
2. Identifying the molecular marker(s) associated with hygienic behaviour traits
3. Determining the association between high hygienic behaviour and tolerance against *Varroa destructor* infestation.

CHAPTER II

REVIEW OF LITERATURE

The literature on screening of honey bee colonies depicting strong hygienic behaviour and or depicting resistance/ tolerance against various brood menaces, and on their genetic characterization including on the molecular basis of their screening is reviewed under various heads and sub-heads as given below:

2.1 Assessment of hygienic behaviour

The bees discard the centre point of infection from the colony, limit disease transmittal within the colony and thus, hygienic behaviour has been linked to resistance to brood disease and in infected colonies it not only provides resistance; but also reduces the spread of diseases and parasitic infections (Rothenbuhler 1964b, Rothenbuhler *et al* 1968, Spivak and Gilliam 1993, 1998a, 1998b). The hygienic behaviour of bees is assessed using different methods of brood killing, like freeze-killed and pin-killed brood (Newton and Ostasiewski 1986, Spivak and Downey 1998).

Boecking and Drescher (1992) observed a correlation between the frozen brood removal and brood infected artificially with *Varroa* females and thus corroborated these results with hygienic bees which removed brood regardless of the cause of damage. Phenotypic selection leads to populations with higher proportions of hygienic bees (Rothenbuhler 1964a, Heath 1982). Palacio *et al* (2000) determined that in a selected population with natural mating, there was change in hygienic behaviour and total hygienic behaviour increased from 66.25 to 84.56 per cent after four years; and brood diseases frequency was lower in hygienic colonies when compared with non-hygienic colonies (Palacio and Bedascarrasbure 2001). Fries and Raina (2003) suggested that an effective hygienic behaviour reported in African sub species *A. mellifera scutellata* Lepeletier, could influence the low level or even absence of American foulbrood in large parts of Africa. Akinwande *et al* (2014) when grouped the colonies of *A. mellifera adansonii* into healthy colonies (HC) and infected colonies (IC) (they show the presence of parasites and brood diseases) and performed hygienic test in both types of colonies; they found that in IC the mean of dead brood removed by the worker honey bees was significantly greater than in the HC.

Hygienic bees remove a greater number of dead brood compared to non-hygienic within the same time frames (Spivak and Gilliam 1993, Kefuss *et al* 1996, Spivak and Downey 1998, Arathi and Spivak 2001, Panasiuk *et al* 2008, Bak *et al* 2010). In hygienic European honey bee colonies, after 6 h of perforation, the dead brood removal ranged between 5-31 per cent in *A. mellifera carnica* (Gramacho and Gonçalves 2009) and reached 64 per

cent in Argentinean honeybee stock, mainly derived from *Apis mellifera ligustica* queens according to Palacio *et al* (2005). In hygienic colonies of Africanized honey bees, the dead brood removal ranged between 7-58 per cent (Morais *et al* 2009). Pereira *et al* (2013) compared the total time spent by workers for removal of dead brood. After one hour of perforation, hygienic bees had removed 43%, while the non-hygienic bees removed only 25%. Hygienic bees completed the removal in 15 h, whereas the non-hygienic bees took over 30 h.

Perez-Sato *et al* (2009) assessed hygienic behaviour by freeze-killed brood method in 31 *A. m. mellifera* colonies to range from 15 to 98 per cent. Sixty five per cent of colonies removed less than 50 per cent of freeze killed brood within 48 h and 22 per cent that removed more than 70 per cent, were thus classified as 'hygienic'. Athreya and Reddy (2013) studied hygienic behaviour variation by pin killed brood method, in *Apis cerana indica* F. in various eco habitats of South India, and revealed that high hygienic response was observed in Kerala followed by Tamil Nadu and the least hygienic behaviour was displayed in the colonies of Karnataka. Honey bee colonies in eco habitats of Kerala were more resistant against the brood diseases and also known for higher honey production, which can be partially attributed to the adaptation against the Thai sacbrood through natural selection via enhanced nest cleaning behaviour.

Rodrigues *et al* (1996) found that colonies removed freeze-killed brood that was recently sealed with a wax capping more quickly than pupae that had been sealed for 5 days. Pereira *et al* (2013) observed that hygienic behaviour response in new combs was significantly more rapid than for brood in older combs. Panasiuk *et al* (2014) evaluated the hygienic behaviour of worker bees with freeze-killed brood in *Apis mellifera carnica* and *A. m. mellifera* and observed that within 24 and 48 hours, these bees removed dead brood above 70 and 80 per cent on average. Toufalia *et al* (2014) quantified hygienic behaviour of *A. mellifera* using the freeze killed brood (FKB) removal assay, and reported that it ranged from 27.5 per cent to 100 per cent in the colonies studied.

Artificial insemination in honey bee is used as an aid in selective breeding as mating cannot be directly controlled in the same manner as in many agriculturally important insects. Colonies comprising of artificially inseminated queens exhibited significantly higher hygiene levels than in the colonies headed by naturally-mated queens. However, the hygiene response in the naturally-mated colonies was encouraging and indicated that providing beekeepers with naturally-mated queens, or virgin queens to mate locally, can result in colonies showing high hygienic response. Bigio *et al* (2014) assessed FKB removal in colonies with artificially inseminated hygienic queens and it ranged between 94.5-100 per cent, indicating that they were all extremely hygienic and 57.8-100 per cent for the naturally mated queens.

Hygienic behaviour was evaluated in 45 production colonies under the Australian honey bee breeding program in Tasmania (Australia) which revealed that 20 per cent of total colonies tested 90-100 per cent hygienic response (mean 96%), 33 per cent colonies tested between 70 and 89 per cent (mean 78%) and 47 per cent tested below 70 per cent (mean 47%) (Anonymous 2015). Rasolofoarivao *et al* (2015) selected 403 *A. m. unicolor* colonies from three regions to observe hygienic responses among them at different time intervals for each hygienic stage and each region. In the first 4 h, on average, colonies had detected at least 50 per cent of the perforated cells. Four hours after perforation, all the colonies displayed some fully uncapped cells. Within 6 h, nine of the tested colonies had uncapped 100% of the perforated cells but none of them had cent per cent brood removal within the 7 h.

2.1.1 Neural mechanisms of hygienic behaviour

Studies have suggested it is the olfactory stimuli that guides in the detection and removal of diseased larvae. A hygienic worker bee has lower threshold level to remove the killed/diseased brood while a non-hygienic bee needs a high threshold level to respond to the stimulus. Thus, the hygienic behaviour expression may be dependent on the perception of appropriate cues as well as on the response threshold of bees to remove those agents releasing such cues. If the abnormal brood (e.g., in the early infection stages with American foulbrood or chalkbrood disease), is still unsealed (larval stage) perforation and uncapping the cell is not required, and thus bees with the greatest olfactory sensitivity may initiate hygienic behavior by removing the larva (Rothenbuhler 1964a, Brodsgaard *et al* 2000).

Individuals varying genetically (hygienic and non-hygienic bees), differ in task performance because they have different thresholds for the stimuli eliciting a task (Robinson and Page 1988). Among the same-aged bees the built-in variation in olfactory sensitivities and responses may result in the tendency for some bees to spend a greater proportion of their time uncapping, and others to spend an equivalent amount of time uncapping and removing (Arathi and Spivak 2001). Various workers demonstrated that within a colony, bees bred for the hygienic trait, exhibit variability in behavioral expression among individual bees, an effect that may be associated with modulatory role of octopamine as the neuromodulator octopamine plays a pivotal role in olfactory-based behaviors of honey bees. Highly hygienic colonies are more sensitive to dead brood odour, and they remove it in a shorter time than others (Arathi *et al* 2000, Masterman *et al* 2001, Spivak *et al* 2002, Gramacho and Spivak 2003, Spivak *et al* 2003, Palacio *et al* 2005).

2.2 Differential hygienic behavior response among different species of *Apis mellifera* and strains

Hygienic behavior is highly variable among and within populations and sub species (Spivak and Gilliam 1998a, Athreya and Reddy 2013). Al-Ghamdi (2002) found that after 24 h, *A.m. yemenitica* removed 100 per cent dead brood while *A.m. carnica* removed only 55 per cent. Kamel *et al* (2003) mentioned that after 24 h, *A.m. lamarckii* removed 72.5per cent of dead brood, while *A.m. carnica* removed 35.6 per cent; after 48 h the two races removed 90.5 and 59.4 per cent, respectively. Al-Medani (2004) reported that after 48 h, *A.m. jemenitica* removed 85.5 per cent. Kefuss (1995) found that *A. m. intermissa* colonies from Tunisia had the highest level of hygienic behaviour as compared with the various sub species of *A. mellifera* (*A. m. mellifera*, *A. m. ligustica*, *A. m. carnica*, *A. m. caucasica*) from France, Tunisia and Chile. Hygienic behaviour did not vary significantly among *A. m. mellifera*, *A. m. carnica*, and 'Buckfast' colonies (Rodriguez *et al* 1996).

Bak *et al* (2010) reported that after 24 h, uncapping behavior of worker bees differed significantly between different lines of *A. mellifera*. The highest percentage of dead brood removal behavior was expressed by mellifera (17%) and Carniolan – Dobra line (13.6%) colonies. The lowest efficiency was observed in the Carniolan Kortowka (Cark) bees (2.0%).

Balhareth *et al* (2012) reported that after 24 h, the mean dead brood removal in *A.m. jemenitica* was significantly higher (85.28%) than that of *A. m. carnica* (43.84%). After 48 h, workers of *A.m. jemenitica* removed 97.60 per cent dead brood, whereas, *A. m. carnica* removed 79.32 per cent; after 72 h, the corresponding brood removal values were 99.78 and 93.29 per cent.

Uzunov *et al* (2014) reported variability for hygienic trait in different genotypes like *A. m. carnica* from Austria and Germany (CarL vs. CarV), *A. m. ligustica* from Italy and Finland (LigI vs. LigF) and *A. m. mellifera* from Denmark and France (MelL vs. MelF). Within *A. m. macedonica* from Macedonia and Bulgaria (MacM vs. MacB) no significant variability observed. Overall, the lowest expression of the trait was observed in MelL, MelF and MacM and the highest in the genotypes (CarV, MacB and CarL). Nouredine and Nizar (2014) conducted hygienic behaviour studies in 40 *A. mellifera intermissa* colonies (Algerian local bees) in spring and fall and showed 91 .56 per cent dead brood removal in spring, and 83.5 per cent in autumn.

Panasiuk *et al* (2014) evaluated the hygienic behaviour of worker bees in relation to *Ascosphaera apis* infected and freeze-killed brood in three races: *A. mellifera carnica* (lines car GR1 and car Mr), *A. m.caucasica* (line cau P), and *A. m. mellifera* (line mel A). The lowest percentage of infected larvae was observed in car GR1 and mel A colonies (8.5% and

15%, respectively) and the highest in car Mr and cau P colonies (21% and 24.3%). Bees in the car GR1 and mel A colonies removed mummified brood in 6.5 and 7.1 days on average, respectively than car Mr and cau P colonies which took above 8 days. Within 24 and 48 h the mel A and car GR1 colonies cleaned significantly more freeze-killed brood (above 70 and 80% on average, respectively) than car Mr and cau P colonies (on average 10 - 20% lower cleaning rate).

2.3 Factors influencing hygienic behaviour

Although hygienic behaviour has positive effects on many characters like honey yield, wax production, royal jelly production, relating to productivity, it is affected by many biotic and abiotic factors. A significant positive correlation between hygienic behaviour and honey production has been reported by Garcia *et al* (2013).

2.3.1 Effect of population size

Although hygienic behavior is genetically determined, its expression depends on colony strength and composition of workers within the colony. Spivak and Gilliam (1993) reported that when the colony strength was altered by changing 10-frame field hives of hygienic and non-hygienic colonies to 2-frame observation hives; it significantly decreased the hygienic response of the hygienic bees, but no affect on the response of the non-hygienic bees was seen. When 20-30% young hygienic bees were added to non-hygienic colonies, the hygienic behaviour response did not increase, but adding young non-hygienic bees to hygienic colonies suppressed the hygienic response. Stanmirovic *et al* (2005) in Syenichko-Peshterski honey bee ecotype analyzed hygienic behaviour of 440 honey bee colonies from 11 localities and reported that hygienic behaviour in populous colonies varied from 95.12 to 99.50 per cent as compared to the weak colonies. Najafgholian *et al* (2011) reported that population size in Iranian honey bees (*A. m. meda*) colonies, had significant effect on the expression of uncapping and removing of infected larvae.

2.3.2 Effect of season

The expression of hygienic behaviour is influenced by season and location as these two interact to yield different combinations of floral availability and nectar flow. Bees remove dead brood faster during a good nectar flow, thereby preparing cells for nectar collection (Thompson 1964, Momot and Rothenbuhler 1971, Robinson 1992, Spivak *et al* 1995, Spivak and Gilliam 1998a, Spivak and Reuter 1998a, Johnson 2003). Whereas, in adverse weather conditions, the activity of foragers reduces, leading to lower pollen and honey stores and subsequently affecting brood rearing and the structure of the bee colony (Mattila and Otis 2006).

Hygienic behaviour decreases with adverse conditions and increases with colony strength during the season. During the active beekeeping season (May-September), Guler and

Toy (2013) evaluated the dead pupae removal behaviour of 90 honey bee colonies and its relationships with the colony productivity. They recorded high brood removal in July and September and lowest in May and August (range: 56.4-99.3%). They also observed significant positive correlations between dead pupae removal and honey yield, bee wax production, and adult worker bee population. Uzunov *et al* (2014) showed that hygienic behaviour was significantly affected by season, location and genetic framework of colony. The highest expression of the trait was recorded in July and August, while the lowest in May and autumn. Tadele *et al* (2014) evaluated hygienic behaviour (June-December) of 28 colonies *Apis mellifera scutellata* by a pin-killed brood assay and observed that it ranged between 86.57 -99.62 per cent, thus, indicating that there was a significant effect of time (month) on performance of these colonies.

Pereira *et al* (2013) analyzed the interaction between hygienic behavior and the period of the day and observed that within the hygienic lines, there were significant differences between periods of the day, implying that although individual workers during the day, from the hygienic strains spent less time uncapping the cells than night, the total time spent on this task was much greater at night than during the day because of the greater number of visits and the longer duration of this behavior at night.

2.4 Hygienic behavior as defense against *Varroa* spp

The *Varroa* mite's natural host is the Asian hive bee, *Apis cerana* Fab. But damage to Asian honey bee colonies is rarely experienced since a stable host-parasite relationship has evolved. (Rath 1999). Such a relationship is distinguishably lacking in the European honey bee, *A. mellifera*. The infestation of *A. mellifera* by *Varroa destructor* Anderson and Trueman was reported to originate nearly half a century ago (Crane 1979, Matheson 1995), when the *A. mellifera* colonies were brought into contact with *A. cerana*.

It is an obligate ectoparasite and feeds on the haemolymph of adult bees and the brood. The capped worker and drone brood of *A. mellifera*, and only the drone brood of *A. cerana* is chosen for reproduction. If timely and proper mite control measures are not undertaken, the mortality in *A. mellifera* colonies due to *V. destructor* can reach up to 100 per cent within two to five years (De Jong 1997). Besides, high mite populations were also observed to be associated with increased incidences of viral infections (Ball 1994), lower weight at emergence, shortened life span of the adult bees (De Jong *et al* 1982), as well as deformed wing and shortened abdomen.

Peng *et al* (1987) found that *A. cerana* uncapped and removed pupae that were infested with the endemic parasitic mite, *Varroa jacobsoni* Oudemans. A significant correlation was found between the infestation level in *A. cerana* and the degree of removal of

infested pupae by the bees. Rath and Drescher (1990) conducted studies in Thailand and reported that *A. cerana* detected and removed 98.8 per cent of worker pupae experimentally infested with *Varroa*. Compared to *A. cerana*, North American and European stocks of *A. mellifera* also removed *Varroa* from infested worker brood but to a limited extent (Boecking 1992, Boecking and Drescher 1991, 1992 and 1993, Boecking and Ritter 1994, Spivak 1996). When *Varroa* was experimentally introduced into *A. m. carnica* colonies in Germany, Boecking and Drescher (1993) found that the mean removal by the Carniolan bees on day 10 after infestation with one mite per cell was 29.3 per cent, 55.1 per cent when two mites per cell were introduced. A positive correlation ($r = 0.74$) was reported between the removal of mite infested brood and the removal of freeze killed brood. During the removal process, the female mite that parasitized the larva may escape and re-enter a different brood cell. Spivak (1996) in the USA tested for their ability to remove infested pupae. The hygienic *A. mellifera* colonies of Starline–Italian origin removed significantly more infested pupae than the non-hygienic colonies 10 days after the brood were infested. Marcangeli (2001) conducted the studies at Coronel Vidal city in the province of Buenos Aires (Argentina), on six *A. mellifera* colonies to determine hygienic response in all colonies using brood perforation method. One week later, 300 *V. jacobsoni* females were introduced in each colony and removal rates were calculated again. All colonies increased their capability to detect and remove dead brood.

The removal of mite-infested brood has been given the term Varroa-sensitive hygienic (VSH) behaviour (Spivak 1996, Ibrahim and Spivak 2006, Harris 2007). Both hygienic and VSH behaviour removed dead or diseased brood, as well as mite-infested brood, but the later is more effective towards mite infestation (Boecking and Spivak 1999, Ibrahim and Spivak 2006, Danka *et al* 2013). The difference between VSH behaviour and regular hygienic behaviour lies in the detection of stimulus of the adult bees; for VSH it is indirect effects of mite infestation such as pupal virus levels or faults in pupal development (Mondet 2014). Whether the mite removal is due to hygienic behaviour or VSH behaviour, most mites are not killed and instead escape during the removal process. Experiments by Boecking and Drescher (1993) revealed that most of the adult female varroa ($n = 104$) that escaped the brood cells after removal could invade other brood cells (mean = 61.3%). Some mites attached themselves to adult bees (14.6%), and a small percentage (10.9%) was killed by bees. If a cell is detected after the female mite has laid eggs on pupa, by the bees her offspring are killed by the bees during the removal of the pupa. Nevertheless, this results in an interruption of the mite's reproductive cycle, which slows down the mite population growth (Boecking and Spivak 1999). The removal of mite-infested brood represents the main factor for the selective breeding of mite tolerant European honey bees (Spivak and Reuter 1998a, Ibrahim and Spivak 2006, Harris 2007). The big difference between the Asian and European

bee species is that the mite is able to reproduce in worker brood cells of *A. mellifera* (Fries *et al* 1994, Boot *et al* 1999) that can lead to colony death within a few years if control measures for mite population are not taken up by beekeepers (Boecking and Genersch 2008).

Hygienic bees removed the majority of mite infested pupal brood at least 60 hours after the cell is capped, which is after the mite has initiated oviposition (Donze *et al* 1996, Spivak 1996). Hygienic behaviour has the potential to limit multiplication of the *Varroa* population in three ways: i) The immature mites are killed when the pupa is removed, which decreases the average number of offspring per reproducing mite, ii) the phoretic period of adult female mites is extended of those mites that survive the removal of the pupae, and iii) the mortality of the adult mites increases if they are damaged by the adult bees through grooming when they escape through the opened cell (Fries *et al* 1994, Spivak 1996); therefore, it has a major influence on the population dynamics of *V. destructor*.

Thakur *et al* (1997) reported that within *A. mellifera* colony, *Varroa* resistance traits are performed at different levels of intensity. They found that Carniolan bees in Germany removed 26-30 per cent of *Varroa*-infested worker brood where various patrines with different distributions of behavioural response thresholds are present. Bienefeld *et al* (2001) observed that 34 of the 80 *Varroa*-infested cells were uncapped during the 12d capped brood phase; 88 per cent during 4th and 7th day after capping. On average 6.8 bees were involved in uncapping of one infested brood cell. 83.7 per cent of the bees were not involved in uncapping, the remaining 16.3 per cent (234 bees) were involved in uncapping of at least one infested brood cell, whereas 0.3 per cent were involved in uncapping of more than 5 (max. 9) cells. The average percentage of bees involved in uncapping was 5.9 per cent in *A.m.mellifera*, 12.5 per cent in the not pure bred *A. m. carnica* colonies (“land-race”) and 19.5 per cent in pure bred *A. m. carnica* colonies and the corresponding values for removing infested brood were 14.7, 24.4 and 32.4 per cent, respectively. The colony effect was highly significant for uncapping and removal of infested brood.

Caspar *et al* (2012) revealed that brood parasitized by virulent mites (i.e. mites with a high potential to induce fatal DWV infections in parasitized pupae) were removed significantly more often than brood parasitized by less virulent mites (i.e. mites with a very low potential to induce overt DWV infections) or non-parasitized brood, thus suggesting that bees showed selective, damage-dependent hygienic behaviour. The hygienic behavior explains 32 per cent of the variance of the mite infestation on bee population, and with the grooming behavior may be sufficient as a mechanism of resistance to *V. destructor* (Xonis *et al* 2015).

Harbo and Hoopingarner (1997) bred bees that maintained low mite levels because on worker brood, the mites had low reproductive success. They found this to be a heritable trait

of the bees, and called it Suppression of Mite Reproduction (SMR). Spivak and Reuter (1998b) observed hygienic behavior response of some colonies containing queens bred for SMR and found that within 48 h, these colonies removed 98.6 per cent of the freeze-killed brood thus indicating that they were expressing hygienic behaviour. In colonies bred for SMR, the mites entered worker brood cells to feed and reproduce; however, the authors reported that the mites died in the cell without reproducing, produced either no progeny, or produced males only, or produced progeny which matured too late (Harbo and Harris, 1999).

Studies in Brazil showed that African bee colonies removed a mean of 51 per cent of the infested brood, while Italian/Africanized hybrid colonies removed 25 per cent. African colonies also removed a significantly more of *Varroa* infested brood than did Italian bee colonies (59 vs. 31%, respectively). Similarly, when African bee colonies were compared with colonies of Italian bees from Fernando de Noronha island near Brazil, the former were found to be significantly more efficient at removing infested brood (61 vs. 35%, respectively) (Guerra 2000).

The negative genetic correlation for hygienic behavior with total reproduction rate of *V. destructor* shows that hygienic behaviour is an important trait for selection. Consequently, in cases of high infestation of *V. destructor*, the selection for hygienic behaviour would decrease the reproduction rate of the mite (Wielewski *et al* 2012). Muli *et al* (2014) in Kenya, reported there was a significant negative correlation between hygienic behaviour and the numbers of *Varroa* in the colonies.

2.4.1 Molecular characterization of hygienic colonies against *Varroa* infestation

The olfactory binding protein gene *OBP3*, a carrier of odorant molecules, is expressed not only in antennae, but also contributes to the olfactory sensitivity of VSH bees. The differential expression of 11 olfactory-related genes (*Trh*, *OR85b-like*, *CSP2*, *NT-7*, *OBP3*, *OBP14*, *GB44647*, *Pyx2*, *GB41230*, *GB50377*, and *GB43812*) suggests that VSH bees express a unusual form of odour-guided behaviour (Foret and Maleszka 2006, Le Conte *et al* 2011). Antennae played a critical role in varroa sensitive hygienic behaviour (VSH) and this was supported by differential gene expression studies conducted in the antennae of VSH and non-VSH (NVS) bees by comparing the antennal transcriptomes of bees that do and do not perform VSH behaviour. Results indicated that antennae play a key role in the expression of VSH behaviour. Comparisons with the antennal transcriptome of nurse and forager bees suggested that VSH profile is more similar to that of nurse bees than foragers (Mondet *et al* 2015).

Several studies also investigated genes associated with hygienic behaviour, or with the trait of resistance to *Varroa* of honey bee colonies. Three studies have used transcriptome analyses (Navajas *et al* 2008, Le Conte *et al* 2011, Gempe *et al* 2012) one study focused on

the proteome (Parker *et al* 2012), and two studies have examined genetic mapping using QTL analyses (Oxley *et al* 2010, Tsuruda *et al* 2012). Of all the genes identified in these studies, 5 genes (*GB52992*, *GB41900*, *GB1356*, *GB55511* and *GB53371*) had functions related to neuronal function or olfactory information processing.

Guarna *et al* (2015) reported that neurosensory detection of odors emitted by dead or diseased larvae is the mechanism behind a complex social immunity behavior that allows bees to co-exist with pathogens as seven putative biomarkers of hygienic behaviour were identified by correlating the expression patterns of individual proteins with hygienic behaviour scores. Intriguingly, these proteins were all involved in semiochemical sensing (odorant binding proteins), nerve signal transmission or signal decay, indicative of the series of events required to respond to an olfactory signal from dead or diseased larvae. They also used recombinant versions of two odorant-binding proteins to identify the classes of ligands that these proteins might be helping bees detect.

Spotter *et al* (2016) conducted genome-wide association study to analyze SNPs and identified six SNP markers that had highly significant associations with detection and uncapping of *Varroa*-parasitized brood by individual worker bees of *A. mellifera*. Putative candidate genes were also discovered after inspection of the genomic regions around these SNPs.

Gene expression analysis at the genomic level between phenotypically selected tolerant and susceptible honey bees to *Varroa* mite revealed that more differentially expressed genes were identified in the pupa stage of the tolerant bees than in bees from the susceptible colony. Based on their molecular functions, the genes were categorized into olfactory signalling, detoxification processes, exoskeleton formation, protein degradation and long chain fatty acid metabolism groups thus suggesting that these biological processes play vital roles in conferring *Varroa* tolerance to naturally selected colonies (Jiang *et al* 2016).

2.5 Genetics of hygienic behaviour

Rothenbuhler (1964b) was the first to conduct genetic studies on hygienic behaviour in 1960s by performing crosses between a strongly hygienic line and a strongly non-hygienic line. The F₁ queens were backcrossed to drones of the hygienic parent and evaluated for hygienic behaviour. The resultant inbred lines were resistant to American foulbrood. In contrast, dead brood remained in the cells of susceptible inbred lines. It was thus, hypothesized under Rothenbuhler's model, that the worker bees that are homozygous at a single recessive 'Uncapping' locus have a high tendency to uncapped brood cells containing diseased pupae. Workers that are homozygous at the 'Removal' locus have a high tendency to remove dead brood from an opened cell. Thus, colonies with a high proportion of workers homozygous at both loci show a high level of hygiene and are resistant to brood diseases.

Rothenbuhler's hypothesis has subsequently been revised to a multiloci model (Moritz 1988, Spivak and Reuter 1998, Lapidge *et al* 2002). Furthermore, the genetic correlation (0.215) between uncapping and removal tasks (Milne 1985) indicated that some loci influence both uncapping and removal tasks. Thus, identification of loci that influence hygienic behaviour would not only provide new insights into how genetic variability can lead to variability in complex behaviour, but would also allow the development of marker-assisted selection for this economically important trait (Oldroyd and Thompson 2007).

2.5.1 Effect of a colony's genotypic composition on the hygienic behaviour expression

A significant increase in the percentage of brood removal was observed with an increase in the percentage of hygienic bees in the colony. Arathi and Spivak (2001) studied that the performance of hygienic behaviour depended on the proportion of hygienic bees in the colony. Hygienic bees in the 25 per cent hygienic colony performed the behaviour well beyond middle age and were more persistent at the task compared with bees from the same genetic line in the other colonies. However, the colony with all worker bees from the hygienic line was more efficient in achieving the task. In the colony with 50 and 100 per cent hygienic bees, the behaviour was partitioned into subtasks, and some bees performed the subtask of uncapping cells at higher frequencies than the subtask of removing dead brood. They also found that the 100 per cent hygienic colony removed dead brood in 12 h. In the same time period the 100 per cent non hygienic colony removed 26.1 per cent and 25 per cent hygienic and 50 per cent hygienic colonies removed 46.2 and 69.6 per cent dead brood, respectively,.

2.5.2 QTLs identified for hygienic behaviour

Lapidge *et al* (2002) identified seven suggestive quantitative trait loci (QTL) for hygienic behaviour and generated a large array of genetic markers using Randomly Amplified Polymorphic DNA (RAPD); each detected QTL controlled only 9–15 per cent of the observed phenotypic variance in the character. Oxley *et al* (2008) confirmed that the markers UBC336 and 395 were correlated with hygienic behaviour in backcross colonies. Oxley *et al* (2010) identified three QTLs that influence the worker bees' propensity to engage in hygienic behaviour and account for up to 30 per cent of the phenotypic variability in hygienic behaviour. Furthermore, they identified two loci influencing the workers to perform uncapping behaviour only, and one locus for the removal behaviour. Behrens *et al* (2011) identified QTLs regulating reduced *Varroa* reproduction using a total of 216 heterozygous microsatellite markers in a bulk segregant analysis. Subsequently, three candidate target regions were fine mapped on chromosomes 4, 7, and 9. The set of all three had significant impact on the suppression of *V. destructor* reproduction by epistasis, although the individual effect of these three QTLs was found to be relatively small,. Tsuruda *et al* (2012) constructed a high-resolution genetic map using 1,340 informative single nucleotide polymorphic (SNP)

markers and interval mapping to analyze the association of the genotypes with the performance of *Varroa* sensitive hygiene. They identified one major QTL on chromosome 9 and a suggestive QTL on chromosome 1.

2.5.3 Genes associated with hygienic behaviour

The genetic basis of the hygienic behavior was further explained by the differential gene expression studies. Navajas (2008) explored differential gene expression of *A. mellifera* associated with *V. destructor* infection comparing parasitized and non-parasitized full-sister pupae from both genetic stocks. They identified a set of 148 genes with significantly different patterns of expression: 32 varied with the presence of *Varroa*, 116 varied with bee genotype, and 2 with both. *Varroa* parasitism caused changes in the expression of genes related to embryonic development, cell metabolism and immunity. Bees tolerant to *Varroa* were mainly characterized by differences in the expression of genes regulating neuronal development, neuronal sensitivity and olfaction. Boutin *et al* (2015) reported genes linked to different regulation patterns (expression level and biological processes) associated with an over-expression of cytochrome P 450 genes; 15 genes were identified with a possible role in transcription regulation. Cytochrome P450 enzymes were over expressed due to disturbance in the regulation of expression induced by changes in transcription regulation or sensitivity to xenobiotics. In turn, these over-expressed cytochrome P450 enzymes degraded the odorant pheromones or chemicals that normally signal the presence of a diseased brood before activation of the removal process, thereby inhibiting hygienic behavior. Gempe *et al* (2016) observed that the colony showing better hygienic behaviour response is achieved by a high number of bees engaging in the hygienic tasks. The percentage of worker bees that engaged in a hygienic behavioural task was three times higher in hygienic colonies as compared to colonies showing low hygienic behaviour thus suggesting that genetic differences may mediate differences in stimulated states to perform hygienic behaviour. 501 differently expressed genes (DEGs) in the brains of hygienic and non-hygienic performing workers in the high hygienic behaviour source bees, and 342 DEGs in the brains of hygienic performing worker bees, relative to the gene expression in non-hygienic worker bees from the low hygienic behaviour source group were identified, 'Cell surface receptor ligand signal transduction' in the high and 'negative regulation of cell communication' in the low hygienic behaviour source were overrepresented molecular processes, suggesting that may play a role in the regulation of quantitative differences in hygienic behaviour.

2.6 Genetic Diversity

The hygienic behaviour expression varies between individual colonies and populations, making it possible to selectively breed bees to enrich hygienic behaviour, with the goal of increasing the ability of the bee population to manage diseases and parasites, and

thus reducing the chemical applications for their control. Testing colonies for selective breeding is a slow and resource-intensive process that could be facilitated by more rapid molecular assays.

In order to determine variation and infer phylogenetic relationships of honey bee populations, many studies have been conducted by using morphometry (Nazzi 1992, Ruttner *et al* 2000), Random Amplified Polymorphic DNA (RAPD) (Hunt and Page 1992, Suazo *et al* 1998), Amplified Fragment Length Polymorphism (AFLP) (Suazo and Hall 1999), allozyme variations (Kandemir *et al* 2000 and 2005, Arias *et al* 2006), RFLP (Restriction Fragment Length Polymorphism) (Suazo and Hall 2002, Szalanski and McKern 2007), mtDNA (Palmer *et al* 2000, Meixner *et al* 2000), microsatellite (Solignac *et al* 2003, Bodur *et al* 2007) and Single Nucleotide Polymorphism (SNP) (Whitfield *et al* 2006). Garnery *et al* (1998) reported that African honey bee populations have very high genetic variation, compared to European populations. It has been also shown that genetic diversity in honey bee colonies increases colony fitness (Mattila and Seeley 2007, Mattila *et al* 2008). Determination of genetic variation of honey bee populations with different markers will provide a valuable resource for selection for desired traits, including tolerance to disease causing agents and ectoparasites, and conservation purposes.

To determine the differences between African and European honeybee populations Suazo *et al* (1998) screened 700 RAPD primers and found five different band patterns each specific for the old world European, new world European, South African and new world African honeybees. Genetic differentiation of Iranian honeybee (*A. m. meda*) populations was also studied with RAPD analysis (Kence *et al* 2005). The percentage of polymorphic loci ranged between 37.14 and 64.76 per cent. Ivanova *et al* (2007) demonstrated genetic variation using RAPD markers, among honey bees in two different mountainous regions and Thrace regions of Bulgaria and Turkey. Twenty Random Amplified Polymorphic DNA (RAPD) primers were used to assess the genetic diversity in 720 worker bees collected from 360 colonies of 25 provinces in Turkey. Ten primers produced 105 reproducible, bright bands; all were polymorphic and mean genetic diversity values ranged between 0.035 and 0.175 (Tunca and Kence 2011).

Munoz *et al* 2009 reported genetic diversity between 9.25 and 9.75 alleles per locus in indigenous populations of *A. m. carnica* and/or *ligustica* in Croatia and Italy. In indigenous Italian and Croatian population, heterozygosity was 0.638 and 0.674, respectively while in US commercial populations Delaney *et al* (2009) reported it to be 0.53. Irene and De La Rua (2012) observed that there was no temporal variation in allelic frequency and heterozygosity between the two surveys conducted in 1998 and 2006. In 1998, the number of alleles per locus ranged from two-seven, whereas they varied from three-seven in 2006.

The mean heterozygosity values observed were 0.437 in 1998 and 0.454 in 2006. The mean number of alleles per *locus* (4.0) and total number of alleles (20) did not vary between 1998 and 2006. Angelique *et al* (2015) investigated a total of 524 *A. mellifera* colonies from 16 beekeepers in 20 sites in Rodrigues, an island in the South-West Indian Ocean with 18 microsatellite markers. The number of detected alleles per locus ranged from 3-15. Over all the sites, the mean number of alleles detected was 7.63. The Rodrigues honeybee population had genetic diversity 8.50 alleles per locus and heterozygosity ($H_{obs}=0.644$).

The response threshold model for division of labor in honey bees (Robinson and Page 1989) explains the enhanced performance among genetically diverse colonies. High genetic diversity among its workers is generated by high levels of multiple mating by its queen. The importation of foreign queens and the practice of moving colonies several times per year are factors that can affect the genetic structure of a local honey bee population through genetic introgression (Garnery *et al* 1998). Honey bee colonies are made up of a large number of patriline ($\sim 10-30$), each patriline comprised of highly related ($r = 0.75$) super sisters headed by the same father. Differences among patrilines show variation in the frequency of task performance and this demonstrates a genetic component responsible for varying task thresholds among patrilines (Robinson 1992, Robinson and Huang 1998). Some of the benefits of this genetic diversity known are improved disease resistance (Hamilton 1987, Tarpy and Seeley 2006, Seeley and Tarpy 2007), stable brood thermoregulation (Jones *et al* 2004), more successful colony founding when compared to genetically similar colonies in which queens are mated with few number of males (Mattila and Seeley 2007) and greater foraging efficiency (Mattila *et al* 2008, Eckholm *et al* 2011).

Munoz *et al* (2008) conducted studies on molecular variability in *A. m. iberiensis* selected for *V. destructor* tolerance where three types of honeybee pupae: pupae not parasitized, infested pupae with mites without reproductive success and infested pupae with mites with reproductive success were genotyped with five microsatellite loci and in the principal coordinate analysis, two main clusters; cluster-1 comprised of 95.9 per cent non-infested pupae and 68.8 per cent infested pupae with mites without reproductive success and 94.1 per cent infested pupae with mites with reproductive success and 31.2 per cent infested pupae with mites without reproductive success in cluster-2.

Thus, the literature available on the studies on hygienic traits of honey bees is highly suggestive that this trait can be used as a selection criterion in queen breeders' apiaries. The selection for hygienic behaviour against infestation of *V. destructor* would reduce the mite population, thus breeding hygienic bees will avoid/reduce the use of chemicals in beekeeping.

The high levels of hygiene shown for the naturally-mated queens present a positive picture for this trait. The presence of genetically diverse populations will enhance the colony performance w.r.t. the economically important behavioural tasks like improved disease resistance, hygienic behaviour, foraging efficiency and nest thermoregulation.

CHAPTER III

MATERIAL AND METHODS

Study of 'Evaluation and molecular characterization of hygienic traits in honey bee (*Apis mellifera* Linnaeus)' was carried out at the *Apis mellifera* Apiary at Entomological Research Farm and Insect Molecular Biology Laboratory, Department of Entomology, and at School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana. The experiments were conducted from February 2015 to April 2017. The material used and methodologies followed for conducting this study is described as given below:

3.1 Screening of honey bee colonies for hygienic traits

The honey bee colonies were screened to study if they manifested variation in their degree of hygienic behaviour. Colony-level hygienic behaviour is usually evaluated by pin killed brood method (Newton and Ostasiewski 1986), measuring the rate at which a colony removes dead pupae that have been killed using a pin. One hundred *A.mellifera* colonies available at the PAU apiary were taken for their assessment for the hygienic behaviour.

3.1.1 Assessment of hygienic behavior

The sealed worker brood at pink eye pupal stage was pierced using No.1size entomological pin (Gramacho *et al* 2003). 100 capped brood cells in one part of the test comb, which was taken from the colony, were perforated just upto midrib of the comb and another 100 cells in the same test comb were used as control. After perforating the brood, the test combs were marked for identifying them to record the observations and were placed back into the colonies. The percentage of brood removal in each colony was recorded after 24, 48 and 72 h. The experiment was run consecutively three times each of 72h duration. Further this experiment was repeated for 3 alternate brood cycles i.e. at every 6 weeks interval between each of the two consecutive test brood cycles. The multiple tests were performed to account for the variability in sub-families (patrilines) w.r.t. their hygienic behaviour.

Determination of the degree of hygienic behaviour (HB) response was calculated as the percentage of dead brood removal after piercing (Newton and Ostasiewski 1986) the brood:

$$HB = \frac{EC(24h) - EC(0h)}{CC(0h)} \times (100 - Z)$$

where, EC (24h) = number of empty cells 24h after perforation; EC(0h)= number of empty cells before perforation; CC (0h)= number of capped cells before perforation, and Z= control correction factor.

$$\text{Control Correction factor } Z = \frac{Y}{A} \times 100$$

where, A = number of capped cells in the control before placing the comb into the colony for the brood removal test; Y = number of new empty cells, calculated as $Y=C-B$; C= number of empty cells after the brood removal test, and B = number of empty cells before the cleaning test. If a colony removed a mean of 80 per cent or more of the dead brood, it was considered hygienic.

The observations recorded at 24, 48 and 72 h included (i) the number of pricked brood cells emptied (dead brood removed), (ii) number of pricked brood cells uncapped but not emptied (dead brood not removed), and (iii) number of pricked brood cells not uncapped. The experiment was laid out in completely randomized block design and data have been analyzed using Analysis of Variance (ANOVA) under factorial CRD for finding the significance of differences among the colonies w.r.t. their hygienic trait, and these were separated by least significant difference (LSD) at $p=0.05$ level (Gomez and Gomez 1984). The data on hygienic behaviour were transformed using arc sine $\sqrt{\text{percentage}}$ transformation before subject to statistical analyses.

3.2 Evaluation of the selected colonies (7 hygienic and 3 non-hygienic) for hygienic behaviour

The selected 10 (7 most hygienic and 3 most non-hygienic) *A. mellifera* colonies from the field studies conducted in preceding experiment were again evaluated for hygienic behaviour as described in § 3.1, to assess their consistency for hygienic traits carried by them in order to take up further molecular studies, and from these colonies samples of adult honey bee workers were collected for molecular characterization of hygienic traits. Thus, the methodology followed, recording of observations and statistical analyses were undertaken as in preceding experiment.

3.3 Molecular characterization of colonies using SSR markers linked with hygienic behaviour

From the colonies identified from field tests as being strongly hygienic (7) or non-hygienic (3), 10 adult worker bees were collected from each of the colonies for each of the three alternate brood cycles i.e. at six weeks interval between each of the two consecutive test brood cycles. Thus, from each colony, 30 samples were collected and in total 300 adult worker honey bees were collected from the ten selected colonies (210 samples from 7 hygienic colonies and 90 samples from 3 non-hygienic colonies) representing the three test brood cycles. The samples were preserved in 100 per cent ethanol and kept at -20°C till DNA was isolated. DNA was isolated from individuals using standardized CTAB method (Evans *et al* 2013). CTAB is two per cent solution of cetyl trimethyl ammonium bromide (CTAB) in 100 mM Tris. Cl (pH= 8.0), which additionally contained 20 mM of Na_2EDTA (pH=8.0), 1.4 M NaCl, 0.5% sodium bisulphite and 1% mercaptoethanol.

3.3.1 DNA isolation

The abdomen and thorax of the individual adult honey bee workers were taken for DNA extraction. Each specimen (decapitated and appendages removed) was taken in a 1.5ml eppendorf tube and macerated individually with a micro pestle in 600 µl of 2X CTAB buffer and 2 µl 2-mercaptoethanol (0.2%). The macerated sample was vortexed for 60 s and later incubated at 65°C for 2 h during which the sample was stirred at regular intervals. Following incubation, 600 µl of chloroform-isoamyl alcohol (24:1) was added to the eppendorf tube and mixed thoroughly on a shaker, followed by spinning at 12,000 rpm for 5 min at 22°C using centrifuge (Gyrogen™). The supernatant was transferred to a similarly marked fresh eppendorf. 10 µl RNase was added and incubated at 37 °C for 50 min. After this treatment, sodium acetate equal to one tenth the volume of supernatant and isopropanol equal to the volume of supernatant was added. The mixture was stirred for 60 s and kept for 20-30 min at -20°C. Then the refrigerated sample was centrifuged at 12,000 rpm for 5 min at 4°C to extract the genomic DNA pellet. The precipitated DNA was rinsed with 500 µl of ethanol (70%). The pellet was first vacuum dried and later air dried by inverting tubes on clean filter paper or by placing the eppendorf in eppendorf-holder under laminar-flow. The DNA was suspended in 100 µl of TE buffer and refrigerated.

3.3.2 Gel electrophoresis

The quality and quantity of DNA isolated from the adult honey bee workers was quantified using Thermo Scientific NanoDrop™ 1000 Spectrophotometer and 0.75 per cent agarose gel (0.75 % agarose gel in 1X TAE buffer and ethidium bromide added @ 1 µg per ml) and the gel was subjected to electrophoresis at 4 volts/ cm for around one hour in 1X TAE buffer. The gels were observed under UV transilluminator and documented using ‘Gel Documentation System’.

The composition of 1X TAE buffer is as under:

Component	Working solution	Quantity per 1,000 ml
Tris base	0.04 M	242 g
Glacial acetic acid	-	57.1 ml
EDTA (0.5M, pH 8.0)	0.001 M	100.0 ml
Sterile water	-	Adjusted to make 1,000 ml of solution

3.3.3 PCR amplification

PCR amplification was performed on each of the 300 genomic DNA samples using five SSR primers reported to be linked with hygienic behaviour (Oxley *et al* 2010), viz. K0263, A058, AT128, AC074 and K1601, the details of which are given in Table 1. PCR

amplification in 10 µl total volume was performed in standard reaction mixture of 2.0 µl of 5X PCR buffer, 0.6-0.8 µl of 25mM MgCl₂, 1.5 µl of 1mM dNTPs, 0.75 µl of each of forward and reverse primer (5 µM), 0.25 µl of Taq DNA polymerase (5 units/µl), 3.0 µl of DNA (60ng) and distilled de-ionized water in 384 wells PCR plates. The PCR programme comprised of initial denaturation for 4 min at 94°C and subsequently denaturation for 1 min at 94°C for the 35 cycles, primer annealing at 48-60°C (varied with the individual SSR primer) for 1min and primer extension at 72°C for 1min. Final extension step was performed for 7 min at 72°C. The PCR products were resolved on 3.0 to 3.5 per cent agarose gel along with standard 50bp DNA ladder (Takara). The agarose gel was run at a constant voltage of 120V at 4v/cm for about 3 h in 0.5X TBE buffer. Gels were observed under UV light and photographed using ‘Gel Documentation System’

3.4 Genetic diversity of colonies using SSR markers

All the genomic DNA samples extracted, as discussed under § 3.3, were used for genotyping the hygienic and non-hygienic colonies using 40 SSR markers (Solignac *et al* 2007), as per the details given in Table 1. PCR amplification was performed on each of the 300 genomic DNA samples (10 worker bees each from each of the 3 brood cycles for each of the 7 most hygienic and 3 most non-hygienic screened out colonies, as described in § 3.3), using 40 SSR primer pairs in 384 wells thermal cycler using standard protocols as described under 3.3, and the PCR products were resolved on 3.0 to 3.5 per cent agarose gel along with standard 50bp DNA ladder (Takara). The agarose gel was run at a constant voltage of 120V at 4v/cm for approximately 3 h in 0.5X TBE buffer. Gels were observed under UV light and the amplification profiles of different SSR primers in the high hygienic and non-hygienic colonies were documented using ‘Gel Documentation System’. Based on the SSR marker presence/absence data, the genetic variability among the colonies was analyzed using software DARwin version 6.0.

Diversity analysis

The genetic diversity among individuals was computed through DARwin (Perrier and Jacquemond-Collet 2006). Polymorphic information content (PIC) value for each of the polymorphic SSR marker was calculated according to the formula:

$$\text{PIC value} = 2f_i(1-f_i)$$

where, f_i is frequency of the amplified allele and $(1-f_i)$ is frequency of null allele.

Diversity index/ Genetic diversity is the expected heterozygosity and was calculated according to formula (Weir 1996) as:

$$\text{DI} = 1 - 1/L \sum_i \sum_j p_i^2$$

where, p_i stands for the frequency of i^{th} allele at 1 locus and L is the number of loci.

Marker index for SSR markers was calculated to determine the capacity of each primer to detect polymorphic loci among the genotypes; MI was calculated according to Powell *et al* (1996) as:

$$MI = DI \times EMR$$

Where, EMR is Effective Multiplex Ratio.

Resolving power (Rp) of primers was calculated according to Prevost and Wilkinson (1999):

$$Rp = \sum IB$$

where IB (band informativeness) is equal to $1 - [2 \times (0.5 - p)]$ and p represents the proportion of genotypes consisting the band.

3.5 Molecular characterization of colonies with the primers designed from the candidate genes for hygienic behaviour

3.5.1 By the presence/absence of candidate genes for hygienic traits

Among the candidate genes for hygienic behaviour identified from QTL studies (Oxley *et al* 2010), 17 genes (Table 2) were selected to perform the molecular characterization of the 10 selected colonies (7 hygienic and 3 non-hygienic) contrasting in hygienic behaviour. Two sets of primers for each of the candidate genes were designed (Table 3) by using IDT PrimerQuest. The PCR amplification of the genomic DNA samples, as in § 3.3, was performed with the 34 newly designed gene specific primers using the same protocol as given in § 3.3 and the PCR products were resolved on 1.5 to 2.0 per cent agarose gel along with standard 100bp DNA ladder (Takara). The agarose gel was run at a constant voltage of 120V at 4v/cm for 3 h in 0.5X TBE buffer. Gels were observed under UV light and the amplification profiles of different gene specific primers in the high hygienic and non-hygienic colonies were photographed using 'Gel Documentation System'.

3.5.2 By sequencing the candidate genes for hygienic traits

Among the seventeen candidate genes studied in the previous experiments, five genes were selected, viz., *GB13839*, *GB12228*, *GB12301*, *GB11135* and *GB19509* for sequencing the amplicons of hygienic and non-hygienic colonies. Four DNA samples each were taken from one most hygienic colony and from one the most non- hygienic colony. The DNA samples were PCR amplified with the primers of the five selected candidate genes in total volume of 50 µl using the same protocol for preparing the mixture of the individual components, as described in § 3.3 and the same PCR programme which comprised of initial denaturation for 4 min at 94°C and for the subsequent 35 cycles, denaturation at 94°C was performed for 1 min, primer annealing temperature (varied with the primer) for 1min and primer extension at 72°C for 1min. Final extension step was performed at 72°C for 7 min, A small volume of 5ul of the PCR product was resolved on 1.5- 2.0 per cent agarose gels along with standard 100bp DNA ladder (Takara) to confirm the amplification of the desired gene

before sending for custom sequencing services from M/s Xcelris Labs, Limited, Ahmadabad (India). Sequences were multiple aligned using 'Gene align function' of the DNA software program 'CLC Free Workbench version 7.5' for determining the natural orientation of each of the sequence among them and also with the reported candidate gene sequence.

3.6 Temporal variation in hygienic behaviour response among the screened out hygienic and non-hygienic colonies

The 10 (7 hygienic and 3 non-hygienic) *A.mellifera* colonies selected from the field studies conducted as described in § 3.1 were evaluated for temporal variation in hygienic behaviour response. The hygienic trait response was assessed by pin killed brood method. The experiment was run consecutively three times each of 72h duration. Further, this experiment was repeated for three alternate brood cycles i.e. at six weeks interval between each of the two consecutive test brood cycles. The observations were recorded at every 2 h interval for three days, on (i) the number of pricked brood cells emptied (dead brood removed), (ii) the number of pricked brood cells uncapped but not emptied (dead brood not removed) and (iii) the number of pricked brood cells not uncapped. The experiment was laid out in completely randomized block design and data have been analyzed using Analysis of Variance (ANOVA) under factorial CRD for finding the significance of differences among the colonies w.r.t. their hygienic trait, and these were separated by least significant difference (LSD) at $p=0.05$ level (Gomez and Gomez 1984). The data on hygienic behaviour were transformed using arc sine $\sqrt{\text{percentage}}$ transformation before subject to statistical analyses.

3.7 Validation of markers identified to be associated with high hygienic behaviour

The queen bees of the 10 selected (7 hygienic and 3 non-hygienic colonies) were taken for genotyping with the five SSR markers reported to be linked with hygienic behaviour (Oxley *et al* 2010). Non-destructive sampling of queen bees was done by taking a wing clip from each of the queen bee of the above 10 colonies for DNA extraction, without causing harm to the queens (Chaline *et al* 2004, Gregory and Rinderer 2004). DNA extraction was done as soon as possible after the specimens' were collected to avoid its degradation and for ensuring best results. The DNA was extracted as per the protocol given in the Nucleospin small tissue DNA purifying kit. The PCR amplification of DNA was done using five SSR primers reported to be linked with hygienic behaviour (Oxley *et al* 2010), viz. K0263, A058, AT128, AC074 and K1601, the details of which are given in Table 1. PCR amplification in 10 μl total volume was performed in standard reaction mixture of 2.0 μl of 5X PCR buffer, 0.6-0.8 μl of 25mM MgCl_2 , 1.5 μl of 1mM dNTPs, 0.75 μl of each of forward and reverse primer (5 μM), 0.25 μl of Taq DNA polymerase (5 units/ μl), 3.0 μl of DNA (60ng) and distilled de-ionized water in 96 wells thermal cycler (Applied Biosystems). The PCR programme comprised of initial denaturation for 4 min at 94°C and in subsequent 35 cycles

each, for 1 min at 94°C, primer annealing at 48-60°C (varied with the individual SSR primer) for 1min and primer extension for 1min at 72°C. Final extension step was performed for 7 min at 72°C. The PCR products were resolved on 3.0 to 3.5 per cent agarose gel along with standard 50bp DNA ladder (Takara) at a constant voltage of 120V at 4v/cm for 3 h in 0.5X TBE buffer. Gels were observed under UV light and documentation done using 'Gel Documentation System'

3.8 Validation of screened hygienic colonies against *Varroa destructor* infestation

The seven hygienic and three non-hygienic colonies were validated against *V. destructor* infestation for the two seasons; autumn and spring. *V. destructor* adult mites were collected from infested worker brood and drone brood using Varroa fork and from adult bees using 'sugar roll method'. The mites were used for inoculating worker larval brood near capping. In total, 21 inoculations (three groups of seven brood cells each) were made in every test colony, thrice in succession. The observations were recorded at every 2h till the complete removal of mite infested brood, on (i) the number of cells uncapped but not emptied, (ii) number of cells uncapped and emptied and (iii) number of cells remaining capped. The time period between inoculation and uncapping of cells and brood removal were also recorded. The experiment was laid out in completely randomized block design and data have been analyzed using Analysis of Variance (ANOVA) under factorial CRD for finding the significance of difference among the colonies w.r.t. their hygienic trait and the colonies were differentiated by least significant difference (LSD) at $p=0.05$ level (Gomez and Gomez 1984). The data on hygienic behaviour were transformed following subject them to $\sqrt{n+1}$ transformation.

Table 1. Details of SSR primers used for genetic diversity analysis of hygienic and non-hygienic populations

S. No.	Name of the marker	Linkage Group	Motif	Size of the allele bp	Forward primer	Reverse primer
1.	K0206	Group2.38	(AT)13	154	ATGCCTAATCATAGATGCTGCAT	TTGAGTCATGGTGTGAACTCTCT
2.	K0264	Group2.39	(AG)15	225	TCTCGTGGAATGGCCTAAGAG	ATACACGCGCACATTCGC
3.	AP098	Group2.39	(CT)7	135	CTCGCGCACCGTACCTTC	GAAGGTTAGGACGGGCGG
4.	K0263	Group2.39	(TA)17	307	TTGTGCAGAAAAAGACAAACACC	TTCTTTACCGCTCACTCGTCTATT
5.	UN368	Group2.40	(TC)8	240	GCAAAAACACATACGCGCA	TAAGTGTCTGTCATCTCGTGAA
6.	SV198	Group2.41	(AG)26	145	TCGACGAATCAACGACGC	ACGTCGAAGCTCATAAATCACG
7.	BI036	Group5.22	(TTA)18	134	AATCCCAACCGCCTGTTG	ATGTAATCACTGCAGAAGTTCTTCTACG
8.	SV172	Group5.22	(AT)10	150	CTCGAGTTTTCTTTATCTATCAGATGTG	TGTTGCATACCTTGACTGCCTAG
9.	A058	Group5.22	(GA)9	85	AAGAATAGATGGACGACGAGAG	GCATCCCACTTGTAAACAATTC
10.	K0519	Group5.22	(AG)15	169	AAGCCGTGTACAGAGTGGGA	ACTCACCTGATATCCGCGC
11.	5983	Group5.22	(AT)10	102	GCGTGCCACCTGTTACG	TCGTGAGTCGGCCGAATC
12.	K0520	Group5.22	(CT)10	191	GTTGCACAGGCACGAGCT	TCCGTTGGAACAAGTTCTCG
13.	K0521	Group5.22	(CT)12	224	CGTCTCGCGATAAACCAGC	TTGAATGTATCGAATCGAATTGG
14.	AC011	Group9.26	(CT)19	127	CTTACGCCAATCTCTCCACG	CGGTTAATTTCTGTTTCTCGC
15.	AT128	Group9.26	(GA)14	186	ACATCGGAAGTGAAAGTCGG	GGCAATCACCGTAAAAGTGAC
16.	BI293	Group9.26	(TC)13	182	GGCGGCTAAACTCACTGGC	TGATCTTGCAGAATTATGCGG
17.	SV026	Group9.26	(AC)8	166	AATCACGGGAAACGTTTGGG	GCACGCAAATTATTCTTGTAAACGTG
18.	AG016	Group9.26	(GT)11	220	GAGAAGTTCACACTCCACTTTACGAG	TCTTAGTTTTCTCCACGCGCC
19.	K0902	Group9.26	(GA)10	156	CCTTCCACGAACACGGAGT	AACTGATATCGAACGGGACGTA
20.	K0901	Group9.26	(GA)8	242	TGCAGATGCGTTCCACCA	CCGATCTCGGTTACCAGGC
21.	5242	Group9.26	(TCTTC)3	316	TGCAGAAATCGACGCGTG	CGCGACGTCAGTCTTGGTATC
22.	K1076	Group10.30	(TC)18	200	AATCACGAGGGAAGCACCTTA	AAAGTGTGAACTCGTGTGAGAA

S. No.	Name of the marker	Linkage Group	Motif	Size of the allele bp	Forward primer	Reverse primer
23.	K1074	Group10.30	(AG)9	201	TATCGATCAGCCCAGCTGC	ACGCAAGTCACGTGGAAGTG
24.	AC074	Group10.31	(CT)5CA(CT)4 ..(CT)6	189	CTCGAGACGCTGAACTCGG	TTCTAACATTGAATCGGGAAAGG
25.	BI234	Group10.31	(TC)11	158	CTACTTGTTGCACGTGCTGATAC	TTCTACGAGCGAGGTGCG
26.	AP146	Group10.31	(AG)25GG (AG)4	151	TCGGATGTTCCGGACCACTTC	GCAGTTCTCCACACCGAGGA
27.	AC109	Group10.31	(CCA)7	119	CAGCGGCACGTTCGACTG	AACATCCGCTCGACCGTG
28.	AC138	Group10.31	(CT)15	164	ACTCGAATATTCATCGCGAAAC	GGCTCCAAGGAGTCTAACGTATC
29.	BI119	Group10.31	(AG)10	172	GACATTCTAAAGAATAATTTTCATCTTTTCG	CGTCTCGATCGTCTCCGG
30.	K1012	Group10.31	(TC)12	163	GCGCAAATAAGAACGTGAAAC	AGCTTGTAAGTGGTGGCGAAG
31.	BI324	Group10.31	(ATA)9	125	ACCGATTGTCTGCTCGCG	AATCGCTTACCGGCAATCC
32.	K1601	Group16.2	(AG)10	177	TCGATCCATCGGTCGTCC	GTGCACCCACGTTACGGTAC
33.	AP217	Group16.2	(CT)10	185	ATCCTATGTGCTCTACAAGTAAACC	GAGCGAAGCTTTCTTGTGC
34.	AP028	Group16.9	(ACAT)AT (ACAT)3	230	CCTGCAACGAGGAGTAACG	GGATGTGGCAAGTTGGAGG
35.	FM2	Group16.9	(AT)10..(AT)9	291	ATTCCCGGTATCATCTCTTG	AATTCGTGGTTAAATTCAAAG
36.	K1610	Group16.9	(AT)10	265	GTAGGTTGATGGCTGCATGC	TGATAAATTCGCACGATAACCG
37.	AP094	Group16.9	(TC)13	133	CGCAACACAACGCATGGT	AAGAGGAGTGGGGTGGGG
38.	K1638	Group16.9	(TC)9	198	TCGATGAACGAGAGAAGCCTT	CGTAATGCGCAATTGCATG
39.	6560	Group16.9	(TC) 11... (AT)10	166	AATTCCAGCCGTCGGTCC	GAAAGTATCCAATATTTTCGCACG
40.	K1656	Group16.9	(TC)22	231	CGTGTTAAACCTCCGAGTGC	ACGATGCGTTTATACGAGTTTCT

Table 2. Details of candidate genes selected for studying the genetic basis of hygienic behaviour

S. No.	Category	Gene ID	Additional associated Gene Ontology definitions
1	Behavioural Modification	GB13839	Long term memory; olfactory learning
2		GB12228	Transcription coactivator activity; circadian regulation of gene expression; synapse assembly; neurotransmitter secretion
3		GB12301	Social behaviour; transcription activator activity
4	Odorant Receptors	GB11135	Odorant binding protein ASP1
5		GB19509	Olfactory behaviour
6	Neuron function/development	GB18541	Transcription factor
7		GB17360	Transcription factor
8	Receptor activity	GB19351	Ecdysone binding
9	Transcription factor/transcription activator activity	GB13500	Transcription factor
10	Receptor binding Neuron function/Development	GB12487	Neuron adhesion; synapse organization; axon extension; nerve maturation; dendrite morphogenesis
11		GB11846	
12	Transcription factor/transcription activator activity	GB17582	Transcription factor
13		GB11352	Pheromone metabolic process; adult feeding behaviour; transcription activator repressor activity
14	Neuron function/development	Abd-A GB19738	Transcription factor activity
15		GB17099	Nervous system development; regulation of transcription
16	Transcription factor/transcription activator activity	GB18285	Transcription factor
17	Receptor activity	LOC413529	RNA binding

(Oxley *et al* 2010)

Table 3: Details of candidate gene specific primers designed by using IDT PrimerQuest Tool

S. No.	Candidate genes	Primer name	Forward primer	Reverse primer	Amplicon (bp)
1.	GB13839	AmP1A	GCAATTTGGGAAAGATGCTAC	AACCGCCAGGATGTAATTC	509
		AmP1B	AGCAGCAAATGCTAGGATAC	GCGTATGTTGAATACGTGGA	509
2.	GB12228	AmP2A	ATGTCCCTGCCCTAATCT	TCTCGCCTGTCCTGTTAT	487
		AmP2B	GGTCCACAACAAGCTACTC	TCGGTTTGACTCGGATTTG	501
3.	GB12301	AmP3A	GGACCGAACGGTTGTATTG	TCCATGACGAGGAGGTAATC	497
		AmP3B	GGGAGGAGGAGGTTAGTTT	GCGTGATGCGATGATTCT	480
4.	GB11135	AmP4A	CTGCCCTGACGAAGAATG	CGCCTCCAACAAGCAATA	503
		AmP4B	GCGTGAAATGAGTCGATGA	CCCAATCCGGCTGAAATAA	474
5.	GB19509	AmP5A	CGCGATTCATCGGTACAA	GTATCGACGGAGGACAAATC	505
		AmP5B	TCTGTCCCTCTCTTTCTCTC	GATCCCATGAAGGCGAATTA	524
6.	GB18541	AmP6A	GAGGAGGAGGAAGAGAAGAA	CACGGGAGGAAACAATAGAG	525
		AmP6B	GGGATATTGGACGGTGATAAA	CGAGATACGAGTCCGAAATG	495
7.	GB17360	AmP7A	ATCGTACGCGATCCTTCTA	TCCTTGAGTATGTCGGTATCT	507
		AmP7B	G TTCATCACCTGGACAACAC	GCTTCCGCATATGGACATAAA	493
8.	GB19351	AmP8A	CCAGCACCGGTATGTTATC	GCACCACGAGTACAATCAT	516
		AmP8B	ACATCAGTGTAGGTGCTAGA	CGTCCATCTGGTCCCTTA	492
9.	GB13500	AmP9A	TCCGCTTCTCGGCTATAA	GCTCTCCTCTTCTCTTCTCT	538
		AmP9B	CCGCTCCCGAAATAGTTTAC	CGGATCGGATAAACGGAAAG	506

S. No.	Candidate genes	Primer name	Forward primer	Reverse primer	Amplicon (bp)
10.	GB12487	AmP10A	TCGAGCATATGCAACTGAATTA	ACAACTAGCACTATCTCCTGT	484
		AmP10B	ATGTCACAAGTCAAAGGTAT	GCTTCACATAACACACTTTC	538
11.	GB11846	AmP11A	AAGTTGCTGCAGGTTCTAC	CACTATGTGGTGAGGGTAATG	519
		AmP11B	AGCCTGAGTAACTGGTCTTA	CTACCAGGTTGTTGGGATATG	495
12.	GB17582	AmP12A	CCTGTGGTAAAGGCTAAGTG	GTTGCTTCGTTCTGGTTAGA	518
		AmP12B	CGTACTTCTCCGGTCCTTAT	GGAATCTGTGCTCTCGTTAC	518
13.	GB11352	AmP13A	CCTCCTTCCCAAAGTCAATC	CTCGTCCTCTTCCTTTCTCT	509
		AmP13B	ACCGTGAGTCGAGTCTATC	CTATCCTCTTTCTCGCGTAATC	503
14.	Abd-A GB19738	AmP14A	GATGGTTGACTGTGGTAGTG	CTCCTATGTGGGCACTTTAC	502
		AmP14B	CGAAGAGAATCGGTGGTATG	ACGAGGAGAGAGAAGAGAAG	487
15.	GB17099	AmP15A	GGAACACGAAGACCTGTATG	GGCACAAACACGAAACATAG	538
		AmP15B	TTTGTGCTGCTCCATGTC	GGATCTTTCTTAGCCCATTGT	497
16.	GB18285	AmP16A	TGCATGTATCGTATGAAGTT	TCTTCTTGGTTCTTCATAGTC	509
		AmP16B	TCTACAACCGATGCAGAAA	CCCAATCTAATTTGACGTTCTT	225
17.	LOC413529	AmP17A	CATGATGATGGACCAGGTTT	TCTTCTTCTGCTCGTCTAGT	543
		AmP17B	GCGCACGAAATATGTCCA	TCTGTTCTGGGTAAAGTAGGA	506

CHAPTER IV

RESULTS AND DISCUSSION

The studies on 'Evaluation and molecular characterization of hygienic traits in honey bee (*Apis mellifera* Linnaeus) were conducted at Punjab Agricultural University, Ludhiana, during 2014-17. During this period, studies conducted included 1) screening of *A. mellifera* colonies for hygienic behaviour, 2) molecular characterization of colonies using SSR markers reported to be linked with hygienic behaviour, 3) candidate gene approach to study if there is sequence variation in candidate genes between hygienic and non-hygienic colonies, 4) temporal variation in hygienic behaviour response among hygienic and non-hygienic colonies, and 5) validation of screened hygienic colonies against *Varroa destructor* infestation. The present study is first of its kind as data about the genetic basis of hygienic behaviour in *A. mellifera* under Indian conditions has been generated, which would serve as baseline for carrying out further molecular studies for hygienic behaviour. Among the 100 colonies screened, 45 per cent of the colonies were hygienic, 42 per cent of the colonies showed intermediary behaviour for hygienic traits and 13 per cent of the colonies were non-hygienic. The sequence variation in gene GB19509 showed six differential SNPs between hygienic and non-hygienic colonies. The results of the above studies have been presented and discussed, in details, as given below.

4.1 Screening of *A. mellifera* colonies for hygienic traits

The colony level hygienic behaviour of 100 colonies was assessed by pin killed brood method which involved measuring the rate at which a colony removes dead pupae that have been killed by pricking with an entomological pin. The experiment was run consecutively three times. Further this experiment was repeated for three alternate brood cycles i.e. at every six weeks interval between each of the two consecutive test brood cycles, to account for any variability w.r.t. the sub-families in the colony.

4.1.1 Dead brood removal at 24, 48 and 72 h of pricking in three alternate brood cycles

In the test colonies, at 24 h following the killing of the capped brood through pin-pricking, the mean brood removed ranged between 31.33-91.33 per cent in brood cycle I, whereas in brood cycle II and III, it ranged between 44.00-92.67 and 55.67-95.00 per cent, respectively. For the three tests brood cycles, the mean pin-killed brood cells found emptied ranged between 46.78-91.00 per cent (Table 4).

After 48 h of the pricking, the mean emptied brood cells in brood cycle I, II and III were found to range between 64.33-95.67, 64.00-95.67 and 70.00-97.67 per cent, respectively; the mean across the three brood cycles ranged between 66.11-94.11 per cent (Table 5).

Table 4. Percentage of brood cells emptied after 24h of pricking in three alternate brood cycles*

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1	66.67 (54.71)	72.67 (58.47)	92.00 (73.73)	77.11 (62.30)	16	65.67 (54.11)	75.00 (60.01)	73.67 (59.11)	71.45 (57.74)	31	40.67 (39.59)	44.00 (41.53)	56.00 (48.4)	46.89 (43.18)
2	71.33 (57.61)	82.00 (64.94)	84.00 (66.40)	79.11 (62.98)	17	81.33 (64.38)	82.00 (64.88)	89.00 (70.61)	84.11 (66.62)	32	89.67 (71.42)	73.67 (59.12)	81.00 (64.13)	81.11 (64.89)
3	72.00 (58.09)	84.33 (66.77)	65.00 (53.71)	73.78 (59.52)	18	83.67 (66.29)	88.33 (70.07)	75.00 (59.98)	82.33 (65.45)	33	85.00 (67.19)	91.00 (72.51)	88.00 (69.75)	88.00 (69.82)
4	78.33 (62.24)	69.67 (56.56)	82.67 (65.37)	76.89 (61.39)	19	86.00 (68.20)	79.33 (62.96)	80.67 (63.89)	82.00 (65.02)	34	63.33 (52.71)	88.00 (69.80)	71.00 (57.40)	74.11 (59.97)
5	83.67 (66.16)	89.33 (70.99)	95.00 (77.42)	89.33 (71.52)	20	62.00 (51.98)	74.67 (59.77)	81.33 (64.38)	72.67 (58.71)	35	61.33 (51.53)	82.67 (65.40)	75.33 (60.20)	73.11 (59.04)
6	71.00 (57.40)	68.00 (55.53)	80.67 (63.95)	73.22 (58.96)	21	89.33 (71.16)	90.00 (71.64)	93.67 (75.40)	91.00 (72.74)	36	74.67 (59.82)	88.67 (70.33)	82.67 (65.40)	82.00 (65.18)
7	86.33 (68.29)	78.00 (62.05)	83.67 (66.14)	82.67 (65.49)	22	80.00 (63.43)	63.33 (52.71)	81.67 (64.63)	75.00 (60.26)	37	71.00 (57.39)	87.33 (69.21)	79.67 (63.18)	79.33 (63.26)
8	60.67 (51.14)	83.00 (65.89)	62.00 (51.92)	68.67 (56.32)	23	87.00 (68.86)	88.00 (69.80)	90.00 (71.59)	88.33 (70.09)	38	82.33 (65.22)	92.67 (74.49)	86.33 (68.33)	87.11 (69.35)
9	78.00 (62.01)	79.67 (63.19)	71.33 (57.61)	76.33 (60.94)	24	80.33 (63.65)	78.00 (62.01)	86.33 (68.31)	81.55 (64.66)	39	74.67 (59.77)	77.67 (61.79)	78.33 (62.24)	76.89 (61.26)
10	78.00 (62.01)	91.67 (73.69)	80.33 (63.65)	83.33 (66.45)	25	91.33 (72.99)	81.00 (64.15)	87.00 (68.85)	86.44 (68.66)	40	31.33 (34.02)	50.67 (45.36)	60.00 (50.75)	47.33 (43.38)
11	69.33 (56.35)	82.33 (65.21)	86.00 (68.00)	79.22 (63.19)	26	75.33 (60.20)	91.67 (73.29)	86.00 (68.09)	84.33 (67.19)	41	77.00 (61.33)	88.33 (70.15)	75.33 (60.20)	80.22 (63.89)
12	81.00 (64.13)	79.67 (63.19)	77.00 (61.32)	79.22 (62.88)	27	52.67 (46.53)	71.67 (57.82)	85.33 (67.46)	69.89 (57.27)	42	85.67 (67.80)	86.67 (68.6)	75.00 (59.98)	82.44 (65.48)
13	78.00 (62.01)	89.67 (71.25)	89.33 (70.98)	85.67 (68.08)	28	66.00 (54.31)	62.66 (54.31)	73.33 (58.89)	68.44 (55.84)	43	71.00 (57.39)	80.67 (63.89)	83.00 (65.68)	78.22 (62.32)
14	69.33 (56.35)	79.33 (62.95)	69.33 (56.35)	72.67 (58.55)	29	74.33 (59.54)	81.00 (64.15)	88.33 (70.00)	81.22 (64.61)	44	39.00 (38.63)	45.67 (42.50)	55.67 (48.23)	46.78 (43.12)
15	87.67 (69.67)	86.33 (68.32)	91.33 (72.86)	88.44 (70.28)	30	75.00 (59.98)	68.00 (55.55)	82.67 (65.38)	75.22 (60.30)	45	66.00 (47.47)	72.00 (58.05)	62.67 (52.32)	66.89 (54.89)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
46	69.33 (56.35)	73.33 (58.91)	76.00 (60.64)	72.89 (58.63)	61	80.67 (63.91)	83.00 (65.65)	85.00 (67.21)	82.89 (65.59)	76	72.33 (58.26)	63.67 (52.91)	68.33 (55.76)	68.11 (55.65)
47	73.00 (58.68)	88.00 (69.80)	67.67 (55.33)	76.22 (61.27)	62	63.00 (52.52)	80.33 (63.68)	84.00 (66.40)	75.78 (60.87)	77	82.67 (65.37)	86.33 (68.28)	60.00 (50.78)	76.33 (61.47)
48	73.67 (59.12)	88.67 (70.47)	88.33 (70.07)	83.56 (66.56)	63	70.00 (56.78)	86.33 (68.31)	88.33 (70.01)	81.56 (65.04)	78	73.33 (48.44)	75.00 (59.98)	72.67 (58.46)	73.67 (59.11)
49	70.33 (57.05)	69.67 (56.56)	70.00 (56.77)	70.00 (56.79)	64	61.67 (51.73)	92.33 (73.92)	74.00 (59.33)	76.00 (61.66)	79	68.67 (55.94)	79.33 (62.95)	63.33 (52.71)	70.44 (57.20)
50	62.00 (51.93)	75.67 (60.43)	71.00 (57.40)	69.56 (56.58)	65	73.67 (59.13)	86.00 (68.06)	84.67 (66.98)	81.45 (64.72)	80	73.33 (58.90)	87.33 (69.13)	73.67 (59.10)	78.11 (62.38)
51	75.00 (60.00)	79.67 (63.18)	79.67 (63.18)	78.11 (62.12)	66	69.00 (56.16)	85.33 (67.47)	90.67 (72.26)	81.67 (65.30)	81	83.33 (66.12)	77.00 (61.33)	70. (56.79)	76.78 (61.41)
52	74.00 (59.32)	73.67 (59.10)	84.33 (66.66)	77.33 (61.69)	67	71.00 (57.40)	83.00 (65.77)	89.33 (71.01)	81.11 (64.72)	82	70.33 (56.98)	70.33 (56.98)	80.33 (63.66)	73.67 (59.21)
53	69.00 (56.14)	69.67 (56.56)	88.00 (69.74)	75.56 (60.81)	68	78.33 (62.26)	71.67 (57.83)	69.33 (56.35)	73.11 (58.81)	83	75.00 (59.98)	79.00 (62.70)	90.33 (71.97)	81.44 (64.89)
54	75.33 (58.24)	86.67 (68.69)	85.00 (67.21)	81.33 (61.26)	69	79.00 (62.70)	79.00 (62.70)	78.00 (62.00)	78.67 (62.47)	84	74.33 (59.54)	81.00 (64.15)	88.33 (70.00)	81.22 (64.61)
55	60.67 (51.15)	76.00 (60.65)	81.00 (64.20)	72.56 (58.66)	70	86.33 (68.29)	67.00 (54.92)	61.00 (51.33)	71.44 (58.18)	85	69.00 (56.16)	72.00 (53.48)	61.00 (51.33)	67.33 (55.17)
56	83.33 (65.93)	81.33 (64.38)	87.33 (69.31)	84.00 (66.54)	71	68.67 (55.95)	70.33 (56.98)	73.00 (58.67)	70.67 (57.20)	86	87.00 (68.85)	86.33 (68.31)	74.67 (59.77)	82.67 (65.64)
57	67.33 (55.13)	85.33 (67.52)	82.67 (65.42)	78.44 (62.69)	72	86.33 (68.29)	82.67 (65.46)	85.33 (67.59)	84.78 (67.11)	87	73.00 (58.67)	70.00 (56.77)	75.00 (59.98)	72.67 (58.47)
58	80.67 (63.90)	80.33 (63.67)	83.00 (65.63)	81.33 (64.45)	73	68.67 (55.95)	80.67 (63.92)	85.00 (67.21)	78.11 (62.36)	88	78.33 (62.84)	77.67 (61.77)	92.00 (73.61)	82.67 (66.07)
59	77.00 (61.33)	80.67 (70.15)	75.33 (60.20)	80.22 (63.89)	74	83.33 (66.12)	82.33 (65.14)	85.67 (67.85)	83.78 (66.37)	89	85.00 (67.27)	81.00 (64.13)	81.00 (64.13)	82.33 (65.18)
60	89.67 (71.42)	73.67 (59.12)	81.00 (64.13)	81.44 (64.89)	75	74.00 (59.35)	75.67 (60.43)	83.33 (65.92)	77.67 (61.90)	90	84.00 (66.93)	88.00 (69.71)	87.33 (69.14)	86.55 (68.59)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
91	89.33 (71.01)	90.67 (72.52)	74.67 (59.82)	84.89 (67.78)
92	73.00 (58.67)	76.33 (60.88)	77.67 (61.77)	75.67 (60.44)
93	75.00 (59.98)	74.67 (59.76)	63.33 (52.71)	71.00 (57.48)
94	86.67 (68.60)	76.00 (60.64)	76.00 (60.64)	79.56 (63.30)
95	80.33 (63.73)	88.67 (70.41)	91.00 (72.53)	86.67 (68.89)
96	86.33 (68.29)	78.00 (62.05)	83.67 (66.14)	82.67 (65.49)
97	80.67 (63.90)	80.33 (63.67)	83.00 (65.63)	81.33 (64.45)
98	86.67 (68.60)	74.33 (59.55)	89.67 (71.29)	83.56 (66.48)
99	80.67 (63.90)	77.00 (61.33)	72.67 (58.47)	76.78 (61.23)
Mean	74.68 (60.13)	78.95 (63.35)	(78.97) (63.49)	

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD ($p=0.05$) for:

Colony	(1.82)
Brood Cycle	(0.32)
Colony x Brood Cycle	(3.15)

Table 5. Percentage of brood cells emptied after 48h of pricking in three alternate brood cycles*

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1	78.67 (62.47)	94.00 (75.82)	93.67 (75.54)	88.78 (71.28)	16	76.00 (60.64)	83.00 (65.66)	95.00 (77.83)	84.67 (68.04)	31	64.33 (53.33)	64.00 (53.12)	70.00 (56.79)	66.11 (54.42)
2	85.00 (67.19)	95.33 (77.51)	95.00 (77.85)	91.78 (74.18)	17	90.00 (71.54)	90.00 (71.54)	90.00 (71.54)	90.00 (71.54)	32	83.33 (65.92)	92.33 (73.95)	94.67 (76.80)	90.11 (72.22)
3	88.33 (70.00)	93.00 (74.70)	87.33 (69.15)	89.55 (71.28)	18	95.33 (77.84)	91.67 (73.23)	83.67 (66.19)	90.22 (72.42)	33	91.33 (72.87)	91.00 (72.51)	94.67 (76.63)	92.33 (74.00)
4	89.00 (70.61)	88.67 (70.31)	87.33 (69.15)	88.33 (70.02)	19	93.33 (75.39)	90.67 (72.22)	88.00 (69.74)	90.67 (72.45)	34	74.33 (59.55)	93.67 (75.46)	82.00 (64.89)	83.33 (66.63)
5	89.33 (70.98)	93.00 (74.65)	96.67 (79.57)	93.00 (75.07)	20	87.00 (69.08)	88.67 (70.30)	89.67 (71.24)	88.44 (70.21)	35	80.33 (63.65)	86.00 (71.21)	88.33 (70.00)	84.89 (67.24)
6	79.33 (62.94)	84.33 (66.67)	87.33 (69.16)	83.66 (66.26)	21	93.67 (75.40)	93.67 (75.67)	95.00 (77.16)	94.11 (76.08)	36	84.00 (66.69)	90.33 (71.99)	93.00 (74.83)	89.11 (71.17)
7	86.33 (68.29)	87.00 (68.97)	87.67 (69.48)	87.00 (68.91)	22	87.67 (69.44)	76.33 (60.92)	86.33 (68.33)	83.45 (66.23)	37	83.00 (69.92)	94.00 (76.00)	90.67 (72.22)	89.22 (71.28)
8	78.33 (62.24)	95.33 (71.61)	84.33 (67.39)	86.00 (69.08)	23	95.67 (78.07)	95.67 (78.07)	90.00 (71.59)	93.78 (75.91)	38	90.33 (71.99)	94.67 (76.67)	91.33 (73.06)	92.11 (73.91)
9	93.67 (75.54)	91.00 (72.81)	82.33 (65.12)	89.00 (71.15)	24	85.00 (71.45)	89.00 (70.60)	91.33 (72.87)	88.44 (71.64)	39	87.33 (69.42)	87.33 (69.30)	85.67 (67.76)	86.89 (68.83)
10	84.00 (66.40)	95.00 (77.16)	87.33 (69.25)	88.78 (70.94)	25	92.33 (74.19)	93.33 (75.02)	93.33 (75.02)	93.00 (74.74)	40	65.67 (54.31)	73.67 (59.11)	79.00 (62.70)	72.78 (58.71)
11	78.00 (60.62)	91.00 (72.53)	90.33 (71.88)	86.44 (68.34)	26	83.66 (66.15)	95.00 (77.16)	89.67 (71.29)	89.44 (71.53)	41	86.33 (68.72)	92.67 (74.37)	87.67 (69.54)	88.89 (70.87)
12	89.67 (71.24)	84.67 (67.27)	94.00 (76.05)	89.44 (71.52)	27	85.00 (68.12)	85.67 (67.80)	91.33 (72.86)	87.33 (69.59)	42	89.67 (71.44)	91.33 (72.95)	87.67 (69.44)	89.56 (71.28)
13	94.00 (76.33)	87.67 (69.51)	89.00 (70.61)	90.22 (72.15)	28	84.67 (67.03)	73.00 (58.68)	85.00 (67.38)	80.89 (64.36)	43	77.00 (61.84)	94.67 (76.67)	92.00 (73.54)	87.89 (70.68)
14	74.33 (59.57)	91.33 (72.86)	74.33 (59.57)	80.00 (64.00)	29	84.00 (66.46)	87.67 (69.67)	89.33 (70.98)	87.00 (69.04)	44	73.00 (58.68)	74.00 (74.37)	72.00 (58.03)	73.00 (58.68)
15	92.00 (74.35)	90.67 (72.26)	97.67 (81.50)	93.44 (76.03)	30	79.67 (63.18)	87.33 (69.45)	90.33 (72.15)	85.78 (68.26)	45	75.00 (57.51)	84.67 (66.95)	84.67 (66.94)	81.44 (64.62)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
46	84.33 (66.73)	89.67 (71.77)	89.00 (70.81)	87.67 (69.77)	61	89.00 (70.81)	90.67 (72.22)	88.00 (69.74)	89.22 (70.92)	76	85.67 (67.98)	90.00 (71.58)	80.33 (63.65)	85.33 (67.74)
47	83.00 (65.72)	94.33 (76.44)	81.00 (64.14)	86.11 (68.77)	62	84.00 (66.42)	91.67 (73.61)	91.67 (73.20)	89.11 (71.08)	77	92.67 (74.31)	95.33 (77.61)	82.67 (65.40)	90.22 (72.44)
48	85.00 (67.19)	91.67 (73.29)	93.33 (75.04)	90.00 (71.84)	63	86.00 (68.01)	91.33 (72.95)	92.00 (73.60)	89.78 (71.52)	78	81.33 (64.38)	87.67 (69.44)	82.67 (65.40)	83.89 (66.41)
49	81.33 (64.47)	84.33 (66.66)	86.67 (68.58)	84.11 (66.57)	64	82.33 (65.12)	95.33 (77.84)	84.67 (66.92)	87.44 (69.96)	79	79.33 (62.96)	92.67 (74.37)	81.67 (64.65)	84.56 (67.32)
50	83.33 (65.89)	88.33 (70.05)	81.00 (52.51)	84.22 (66.69)	65	81.00 (64.26)	91.00 (72.56)	90.33 (71.86)	87.44 (69.56)	80	81.33 (64.38)	92.67 (74.37)	86.00 (68.02)	86.67 (68.93)
51	83.67 (66.19)	86.33 (68.41)	86.33 (68.41)	85.44 (67.67)	66	74.67 (59.79)	92.00 (73.61)	93.00 (74.70)	86.56 (69.36)	81	91.33 (73.01)	87.33 (69.13)	83.00 (65.68)	87.22 (69.27)
52	79.67 (63.19)	89.33 (70.99)	87.33 (69.13)	85.44 (67.77)	67	78.33 (62.26)	89.00 (70.60)	93.33 (75.21)	86.89 (69.36)	82	83.00 (65.62)	83.00 (65.62)	85.67 (67.74)	83.89 (66.33)
53	84.00 (66.40)	83.67 (66.19)	90.00 (71.54)	85.89 (68.04)	68	82.00 (64.87)	88.33 (70.00)	84.00 (66.42)	84.78 (67.10)	83	87.00 (68.88)	87.00 (68.88)	93.00 (74.70)	89.00 (70.82)
54	86.00 (68.01)	91.33 (72.95)	92.00 (73.60)	89.78 (71.52)	69	85.00 (67.22)	91.33 (72.99)	91.00 (72.51)	89.11 (70.91)	84	84.00 (66.46)	87.67 (69.67)	89.33 (70.98)	87.00 (69.04)
55	89.33 (70.93)	88.33 (71.57)	87.67 (69.43)	88.44 (70.12)	70	96.33 (78.95)	81.67 (64.70)	80.33 (63.65)	86.11 (69.10)	85	88.33 (70.00)	85.33 (67.54)	77.00 (61.36)	83.55 (66.30)
56	89.00 (70.60)	82.67 (65.37)	92.67 (74.60)	88.11 (70.19)	71	81.00 (64.26)	80.67 (63.90)	85.00 (67.22)	82.22 (65.12)	86	90.33 (71.86)	90.33 (71.86)	83.67 (66.14)	88.11 (69.95)
57	81.00 (64.15)	92.00 (73.61)	89.33 (70.91)	87.44 (69.56)	72	87.00 (68.88)	87.00 (68.88)	93.00 (74.70)	89.00 (70.82)	87	82.00 (64.89)	81.00 (64.14)	81.00 (64.14))	81.33 (64.39)
58	89.00 (71.02)	92.00 (73.70)	90.00 (71.54)	90.33 (72.09)	73	81.00 (64.26)	91.00 (72.56)	90.33 (71.86)	87.44 (69.56)	88	90.00 (71.58)	88.33 (70.00)	95.00 (77.05)	91.11 (72.88)
59	86.33 (68.72)	92.67 (74.37)	87.67 (69.54)	88.89 (70.87)	74	87.67 (69.42)	87.33 (69.30)	85.67 (67.76)	86.89 (68.83)	89	88.33 (70.28)	86.33 (68.29)	86.33 (68.29)	87.00 (68.95)
60	93.67 (75.46)	87.67 (69.51)	87.00 (68.87)	89.44 (71.28)	75	82.33 (65.34)	88.00 (69.71)	93.00 (74.65)	87.78 (69.90)	90	89.00 (70.60)	94.67 (76.63)	90.00 (71.58)	91.22 (72.94)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
91	94.00 (75.92)	94.00 (75.92)	82.67 (65.38)	90.22 (72.41)
92	82.00 (64.89)	92.00 (73.85)	84.67 (66.94)	86.22 (68.56)
93	83.00 (65.62)	94.33 (76.28)	77.67 (61.79)	85.00 (67.90)
94	89.67 (71.25)	90.00 (71.54)	90.00 (71.54))	89.89 (71.44)
95	92.00 (75.05)	93.00 (74.83)	93.00 (74.63)	92.67 (74.83)
96	86.33 (68.29)	87.00 (68.97)	87.67 (69.48)	87.00 (68.91)
97	89.00 (71.02)	92.00 (73.70)	90.00 (71.54)	90.33 (72.09)
98	89.67 (71.25)	93.67 (75.58)	93.67 (75.58)	92.33 (74.14)
99	89.00 (71.02)	88.00 (69.80)	78.33 (62.24)	85.11 (67.69)
Mean	85.08 (67.76)	89.00 (71.13)	87.75 (69.99)	87.66 (69.63)

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

Colony	(2.39)
Brood Cycle	(0.42)
Colony x Brood Cycle	(4.14)

The data in Table 6 shows that the mean pin killed brood removal ranged between 70.67-95.67, 77.33-95.67 and 75.00-96.67 per cent in brood cycle I, II and III, respectively. The overall mean brood cells emptied ranged between 74.33-94.89 per cent.

4.1.2 Dead brood cells only uncapped after 24, 48 and 72 h of pricking in three alternate brood cycles

Data in Table 7 revealed that after 24 h of pricking of the brood, of the first test brood cycle, the mean percentage of pricked brood cells only uncapped ranged between 0.00-31.33 per cent, in the alternate second test brood cycle, it was 0.00-37.33 per cent, and in third alternate test brood cycle, it ranged between 0.00-26.33 per cent. The overall mean of pin killed brood cells which were only uncapped, over the three brood cycles, ranged between 1.44 to 22.22 per cent.

After 48 h of the treatment, in brood cycle I, the maximum pin killed brood cells only uncapped was 20.67 per cent, while in brood cycle II and III the maximum was 12.33 and 11.33 per cent, respectively. The mean over the brood cycles, ranged between 0.44-10.44 per cent among the screened colonies (Table 8).

In the first test brood cycle, after 72 h of the killing of the brood (Table 9), the maximum brood cells that were only uncapped were 11.00 per cent, whereas in the second and third alternate test brood cycles, the corresponding figures were 11.67 and 15.33 per cent, respectively. The overall mean over the three brood cycles, only uncapped brood cells ranged between 0.00-11.67 per cent.

4.1.3 Dead brood remaining capped after 24, 48 and 72 h of pricking in three alternate brood cycles

In the first brood cycle, the pin killed brood cells remaining capped after 24 h of pricking ranged between 0.00-59.33 per cent. In the second and third alternate test brood cycles, the brood cells remaining capped were in the range of 2.33-47.33 and 3.67-33.33 per cent. The overall mean (over the brood cycles) of pin killed brood cells remaining capped ranged between 6.00-43.56 per cent (Table 10).

After 48 h of treatment (Table 11), the brood cells remaining capped ranged between 2.33-31.00 per cent in first test brood cycle. In second alternate brood cycle tested, the brood cells remaining capped were in the range of 2.00-34.00 per cent and in third alternate test brood cycle, it ranged between 2.33-28.00 per cent. The overall mean of brood cells remaining capped, were reduced to minimum of 4.22 and a maximum of 31.00 per cent across the screened colonies.

The data recorded after 72 h of treatment (Table 12) depicted that in the first test brood cycle, the brood cells remaining capped were in the range of 1.33-26.00 per cent whereas in second and third alternate brood cycles evaluated, it ranged between 0.67-22.67 and 1.00-20.33 per cent, respectively. The mean percentage of pricked brood cells remaining capped, across all the three brood cycles, were in the range of 2.11-23.00 per cent.

Table 6. Percentage of brood cells emptied after 72h of pricking in three alternate brood cycles*

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1	81.67 (64.62)	94.00 (75.82)	95.00 (77.05)	90.22 (72.50)	16	79.00 (62.70)	87.67 (69.57)	95.67 (78.60)	87.44 (70.29)	31	70.67 (57.20)	77.33 (61.57)	75.00 (59.99)	74.33 (59.59)
2	86.00 (68.01)	95.33 (77.51)	95.00 (77.85)	92.11 (74.46)	17	92.00 (73.54)	92.00 (73.54)	92.00 (73.54)	92.00 (73.54)	32	86.00 (68.08)	92.67 (74.29)	94.67 (76.80)	91.11 (73.06)
3	89.00 (70.61)	93.00 (74.70)	90.00 (71.59)	90.67 (72.30)	18	96.33 (79.01)	92.33 (73.90)	85.67 (67.82)	91.44 (73.58)	33	92.33 (73.90)	91.67 (73.22)	94.67 (76.63)	92.89 (74.58)
4	89.67 (71.22)	92.33 (73.99)	87.33 (69.15)	89.78 (71.45)	19	96.00 (78.49)	91.00 (72.58)	89.67 (71.22)	92.22 (74.10)	34	79.33 (62.94)	93.67 (75.46)	87.33 (69.15)	86.78 (69.18)
5	90.33 (71.92)	93.67 (75.53)	96.67 (79.57)	93.55 (75.67)	20	89.33 (71.08)	91.33 (72.92)	91.67 (73.20)	90.78 (72.40)	35	83.67 (66.17)	86.67 (68.61)	92.67 (74.31)	87.67 (69.70)
6	79.33 (62.94)	88.67 (70.33)	92.33 (74.02)	86.78 (69.10)	21	93.67 (75.40)	93.67 (75.67)	95.00 (77.16)	94.11 (76.08)	36	87.67 (69.79)	91.00 (72.70)	94.33 (76.28)	91.00 (72.92)
7	86.33 (68.29)	91.00 (72.91)	92.33 (73.90)	89.89 (71.70)	22	87.67 (69.44)	81.00 (64.15)	90.00 (71.63)	86.22 (68.41)	37	83.00 (70.93)	94.00 (65.62)	93.00 (74.71)	90.00 (72.11)
8	81.67 (64.63)	90.00 (71.73)	95.33 (74.65)	89.00 (71.32)	23	95.67 (78.07)	95.67 (78.07)	93.33 (75.21)	94.89 (77.12)	38	93.67 (75.43)	94.67 (76.67)	92.67 (74.40)	93.67 (75.50)
9	94.33 (76.52)	93.67 (75.58)	94.00 (75.79)	94.00 (75.96)	24	85.00 (67.19)	89.00 (70.60)	93.67 (75.46)	89.22 (71.08)	39	87.67 (69.42)	89.67 (71.30)	88.33 (70.22)	88.56 (70.31)
10	84.00 (69.40)	95.33 (77.97)	91.33 (72.89)	90.33 (72.42)	25	94.33 (76.67)	94.67 (76.63)	93.33 (75.02)	94.11 (76.10)	40	73.33 (59.15)	79.67 (63.18)	83.33 (65.88)	78.78 (62.74)
11	82.00 (64.88)	92.00 (73.56)	93.00 (74.63)	89.00 (71.02)	26	89.00 (66.15)	95.00 (77.16)	91.67 (73.47)	90.11 (72.26)	41	87.67 (70.06)	93.67 (75.54)	93.00 (74.73)	91.44 (73.44)
12	91.33 (72.86)	87.33 (69.31)	95.00 (77.22)	91.22 (73.13)	27	86.67 (69.30)	87.00 (68.87)	94.00 (75.88)	89.22 (71.35)	42	92.00 (73.79)	92.33 (73.99)	91.00 (72.53)	91.78 (73.44)
13	94.00 (76.33)	91.00 (72.81)	89.00 (70.61)	91.33 (73.25)	28	91.00 (72.57)	77.67 (63.76)	86.00 (68.12)	84.89 (67.49)	43	81.33 (64.97)	94.67 (76.67)	92.00 (73.54)	89.33 (71.73)
14	82.67 (65.43)	93.33 (75.07)	82.67 (65.43)	86.22 (68.64)	29	86.33 (68.33)	88.33 (70.21)	91.33 (72.86)	88.67 (70.47)	44	86.33 (68.31)	81.33 (64.38)	80.00 (63.41)	82.55 (65.37)
15	94.00 (76.33)	91.67 (73.22)	97.67 (81.50)	94.44 (77.01)	30	79.67 (63.18)	88.00 (69.95)	93.00 (74.87)	86.89 (69.33)	45	81.00 (64.14)	89.67 (71.24)	84.67 (66.94)	85.11 (67.44)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
46	87.33 (69.19)	91.33 (73.22)	90.67 (72.26)	89.78 (71.56)	61	92.00 (73.61)	92.00 (73.56)	91.00 (72.51)	91.67 (73.23)	76	88.67 (70.31)	91.00 (72.51)	84.00 (66.42)	87.89 (69.75)
47	87.00 (69.00)	94.33 (76.44)	83.67 (66.14)	88.33 (70.53)	62	86.00 (68.01)	93.67 (75.67)	93.67 (75.54)	91.11 (73.07)	77	94.00 (75.82)	95.00 (77.19)	89.00 (70.64)	92.67 (74.55)
48	89.67 (71.22)	91.67 (73.29)	94.00 (75.79)	91.78 (73.43)	63	89.33 (70.99)	92.00 (73.83)	92.00 (73.60)	91.11 (72.81)	78	83.33 (65.89)	91.00 (72.57)	88.33 (70.01)	87.55 (69.49)
49	86.00 (68.06)	88.00 (69.71)	91.33 (72.86)	88.44 (70.21)	64	82.33 (65.12)	96.00 (78.59)	84.67 (66.92)	87.67 (70.21)	79	87.33 (69.19)	94.33 (76.34)	85.67 (67.78)	89.11 (71.10)
50	84.33 (66.67)	91.33 (72.99)	84.00 (66.40)	86.55 (68.69)	65	84.33 (66.74)	91.67 (73.20)	91.00 (72.57)	89.00 (70.83)	80	83.33 (68.12)	93.33 (75.21)	90.67 (72.26)	89.11 (71.12)
51	85.33 (67.52)	93.00 (74.65)	93.00 (63.90)	90.44 (72.27)	66	82.33 (65.14)	93.00 (74.70)	93.67 (75.40)	89.67 (71.30)	81	91.33 (73.01)	89.67 (71.24)	85.33 (67.46)	88.78 (70.57)
52	86.67 (68.74)	90.67 (72.34)	91.67 (73.20)	89.67 (71.42)	67	82.00 (64.89)	90.33 (71.94)	93.33 (75.21)	88.56 (70.68)	82	83.00 (65.62)	83.00 (65.62)	93.00 (74.71)	86.33 (68.65)
53	85.33 (67.46)	87.00 (68.87)	90.67 (72.20)	87.67 (69.51)	68	87.00 (68.84)	91.00 (72.53)	89.00 (70.64)	89.00 (70.67)	83	88.33 (70.06)	91.00 (72.58)	93.33 (75.04)	90.89 (72.56)
54	89.33 (70.99)	92.00 (73.83)	92.00 (73.60)	91.11 (72.81)	69	88.67 (70.33)	92.67 (74.27)	93.00 (74.63)	91.45 (73.07)	84	86.33 (68.33)	88.33 (70.21)	91.33 (72.86)	88.67 (70.47)
55	90.00 (71.59)	88.33 (70.00)	90.33 (71.86)	89.55 (71.15)	70	96.33 (78.95)	87.33 (69.33)	87.00 (68.84)	90.22 (71.67)	85	88.33 (70.00)	88.67 (70.51)	84.00 (66.41)	87.00 (68.97)
56	91.00 (72.53)	84.67 (66.92)	93.67 (75.54)	89.78 (71.66)	71	84.33 (66.74)	85.00 (67.19)	90.33 (71.89)	86.56 (68.61)	86	90.33 (71.86)	90.33 (71.86)	86.00 (68.00)	88.89 (70.57)
57	85.67 (67.74)	92.67 (74.49)	89.67 (71.24)	89.33 (71.15)	72	88.33 (70.06)	91.00 (72.58)	93.33 (75.04)	90.89 (72.56)	87	88.00 (69.75)	88.67 (70.35)	88.67 (70.35)	88.45 (70.15)
58	91.33 (73.06)	92.00 (73.70)	92.00 (73.60)	91.78 (73.45)	73	84.33 (66.74)	91.67 (73.20)	91.00 (72.57)	89.00 (70.83)	88	91.00 (72.51)	88.33 (70.00)	95.00 (77.05)	91.44 (73.19)
59	87.67 (70.06)	93.67 (75.54)	93.00 (74.73)	91.44 (73.44)	74	87.67 (69.42)	89.67 (71.30)	88.33 (70.22)	88.56 (70.31)	89	88.33 (70.28)	86.33 (68.29)	86.33 (68.29)	87.00 (68.95)
60	93.67 (75.46)	91.33 (72.97)	89.00 (70.60)	91.33 (73.01)	75	86.00 (68.18)	89.67 (71.28)	94.33 (76.21)	90.00 (71.89)	90	91.00 (72.51)	93.33 (75.12)	90.00 (71.58)	91.44 (73.07)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
91	94.00 (75.92)	94.00 (75.92)	87.67 (69.43)	91.89 (73.76)
92	88.00 (69.75)	92.00 (73.85)	85.67 (67.73)	88.56 (70.44)
93	88.00 (69.71)	94.33 (76.28)	86.33 (68.30)	89.56 (71.43)
94	89.67 (71.25)	90.00 (71.54)	90.00 (71.54)	89.89 (71.44)
95	93.67 (76.37)	93.33 (75.17)	93.67 (75.40)	93.56 (75.65)
96	86.33 (68.29)	91.00 (72.91)	92.33 (73.90)	89.89 (71.70)
97	91.33 (73.06)	92.00 (73.70)	92.00 (73.60)	91.78 (73.45)
98	89.67 (71.25)	93.67 (75.58)	93.33 (75.19)	92.22 (74.01)
99	91.33 (73.06)	90.67 (72.26)	80.33 (63.65)	87.44 (69.66)
Mean	87.52 (69.74)	90.72 (72.62)	90.46 (72.37)	89.57 (71.57)

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) in

Colony (2.21)

Brood Cycle (0.38)

Colony x Brood Cycle (3.82)

Table 7. Percentage of brood cells only uncapped after 24h of pricking in three alternate brood cycles*

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1	15.00 (22.78)	22.33 (28.15)	2.33 (6.64)	13.22 (19.19)	16	13.00 (21.13)	16.33 (23.80)	22.33 (28.13)	17.22 (24.35)	31	9.33 (17.70)	8.67 (17.09)	10.67 (18.98)	9.56 (17.92)
2	13.67 (21.68)	15.67 (23.24)	9.00 (17.38)	12.78 (20.77)	17	0.00 (0.00)	3.00 (9.97)	3.00 (9.88)	2.00 (6.62)	32	4.33 (11.76)	13.33 (21.37)	9.67 (18.09)	9.11 (17.07)
3	4.33 (11.23)	8.67 (17.00)	26.33 (30.86)	13.11 (19.70)	18	4.00 (11.53)	3.00 (9.72)	7.33 (15.56)	4.78 (12.27)	33	3.00 (9.72)	0.00 (0.00)	6.67 (14.92)	3.22 (8.22)
4	11.67 (19.96)	25.00 (29.97)	2.00 (8.13)	12.89 (19.35)	19	4.67 (11.56)	10.67 (19.05)	10.00 (18.32)	8.44 (16.31)	34	17.00 (24.32)	3.67 (8.80)	13.67 (21.68)	11.44 (18.27)
5	0.33 (1.91)	2.67 (7.63)	1.33 (5.42)	1.44 (4.99)	20	12.67 (20.84)	11.67 (19.82)	8.67 (17.01)	11.00 (19.22)	35	20.67 (27.03)	4.00 (11.47)	8.33 (16.77)	11.00 (18.42)
6	16.00 (23.54)	21.67 (27.73)	12.67 (20.82)	16.78 (24.03)	21	2.33 (7.01)	4.67 (12.28)	2.00 (6.55)	3.00 (8.62)	36	10.33 (18.62)	1.00 (3.32)	7.67 (15.94)	6.33 (12.63)
7	2.00 (6.65)	8.67 (16.98)	3.67 (10.86)	4.78 (11.49)	22	17.67 (24.84)	20.00 (26.55)	4.00 (11.28)	13.89 (20.89)	37	10.33 (18.43)	2.00 (6.65)	10.67 (19.04)	7.67 (14.71)
8	23.00 (28.64)	14.33 (22.22)	29.33 (32.78)	22.22 (27.88)	23	4.00 (11.37)	3.00 (8.05)	0.67 (3.82)	2.56 (7.75)	38	6.67 (14.95)	0.67 (2.71)	6.67 (14.85)	4.67 (10.83)
9	8.00 (16.42)	8.00 (16.29)	13.00 (21.13)	9.67 (17.95)	24	4.00 (11.32)	9.00 (17.40)	4.33 (11.99)	5.78 (13.57)	39	16.00 (23.55)	6.67 (14.89)	7.67 (16.02)	10.11 (18.15)
10	8.00 (16.42)	3.67 (10.48)	8.00 (16.40)	6.56 (14.43)	25	3.67 (11.01)	1.33 (3.84)	0.00 (0.00)	1.67 (4.95)	40	9.33 (17.70)	7.33 (15.65)	22.67 (28.40)	13.11 (20.58)
11	16.00 (23.55)	3.67 (9.03)	7.00 (15.34)	8.89 (15.97)	26	9.67 (18.10)	0.00 (0.00)	3.33 (8.57)	4.33 (8.89)	41	7.00 (15.27)	7.00 (15.27)	13.67 (21.67)	9.22 (17.40)
12	19.00 (25.83)	8.67 (16.95)	16.00 (23.54)	14.56 (22.11)	27	31.33 (33.82)	10.00 (18.38)	9.00 (17.43)	16.78 (23.21)	42	5.33 (12.93)	6.67 (14.85)	13.33 (21.39)	8.44 (16.39)
13	0.00 (0.00)	3.00 (9.97)	1.67 (6.03)	1.56 (5.33)	28	28.00 (31.94)	14.00 (21.96)	7.67 (16.02)	16.56 (23.30)	43	17.00 (24.34)	6.67 (14.89)	2.00 (7.94)	8.56 (15.73)
14	22.33 (28.16)	15.33 (23.01)	22.33 (28.16)	20.00 (26.44)	29	3.33 (8.61)	1.33 (5.42)	1.00 (3.32)	1.89 (5.78)	44	5.00 (12.80)	37.33 (37.64)	20.33 (26.78)	20.89 (25.74)
15	4.67 (12.41)	5.00 (12.87)	2.00 (7.94)	3.89 (11.08)	30	8.33 (16.77)	19.67 (26.31)	6.33 (14.530)	11.44 (19.20)	45	16.00 (23.55)	16.33 (23.82)	16.00 (23.52)	16.11 (23.63)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
46	17.67 (24.81)	8.00 (16.17)	11.67 (19.92)	12.44 (20.30)	61	4.00 (10.89)	5.00 (12.87)	0.67 (2.71)	3.22 (8.83)	76	10.67 (18.96)	9.33 (17.76)	20.00 (26.54)	13.33 (21.08)
47	10.00 (18.43)	9.33 (17.76)	10.33 (18.71)	9.89 (18.30)	62	19.00 (25.82)	7.00 (15.23)	9.00 (17.45)	11.67 (19.50)	77	7.00 (15.06)	8.67 (17.11)	11.33 (19.66)	9.00 (17.28)
48	13.33 (21.41)	2.00 (6.55)	4.33 (9.78)	6.55 (12.58)	63	2.00 (8.13)	7.00 (2.71)	2.00 (6.65)	3.67 (10.03)	78	20.33 (26.77)	3.67 (8.80)	19.33 (26.05)	14.44 (22.54)
49	12.00 (14.96)	18.33 (25.31)	19.00 (25.83)	16.44 (23.80)	64	22.67 (28.41)	4.33 (11.99)	11.67 (19.95)	12.89 (20.12)	79	20.33 (26.78)	16.33 (37.64)	17.33 (27.02)	18.00 (25.02)
50	19.33 (26.07)	11.33 (19.61)	15.00 (22.77)	15.22 (22.81)	65	14.67 (22.51)	9.67 (18.04)	4.67 (12.16)	9.67 (17.57)	80	3.67 (8.80)	3.67 (13.75)	20.33 (26.79)	9.22 (14.80)
51	5.33 (13.26)	13.00 (21.09)	13.00 (21.09)	10.44 (18.48)	66	13.33 (21.37)	6.33 (14.50)	0.00 (0.00)	6.55 (11.96)	81	27.00 (31.29)	13.00 (21.13)	12.67 (20.82)	17.56 (24.41)
52	4.00 (11.53)	11.67 (19.94)	1.67 (7.15)	5.78 (12.87)	67	14.00 (21.96)	1.33 (3.840)	1.33 (6.53)	5.55 (10.78)	82	5.33 (13.33)	10.67 (19.05)	6.00 (13.86)	7.33 (15.42)
53	17.00 (24.34)	2.67 (9.36)	2.67 (7.69)	7.44 (13.79)	68	11.67 (19.88)	13.00 (21.12)	9.33 (17.75)	11.33 (19.58)	83	7.67 (15.77)	8.67 (16.98)	4.67 (12.41)	7.00 (15.05)
54	16.67 (24.08)	6.00 (14.08)	3.00 (9.97)	8.56 (15.05)	69	12.00 (20.22)	11.33 (19.66)	9.00 (17.45)	10.78 (19.11)	84	3.33 (8.61)	1.33 (5.42)	1.00 (3.32)	1.89 (5.78)
55	10.67 (19.05)	13.67 (11.99)	2.00 (6.65)	8.78 (15.79)	70	10.00 (18.39)	13.67 (21.68)	16.33 (23.82)	13.33 (21.30)	85	18.67 (25.56)	14.67 (22.51)	9.67 (17.99)	14.33 (22.02)
56	7.67 (16.04)	4.00 (11.47)	4.67 (12.41)	5.45 (13.31)	71	18.33 (25.33)	14.00 (21.94)	15.33 (22.96)	15.89 (23.41)	86	1.67 (6.03)	4.00 (11.37)	6.33 (14.56)	4.00 (10.65)
57	3.67 (10.95)	1.33 (3.84)	6.00 (14.07)	3.67 (9.62)	72	10.00 (18.39)	4.33 (11.99)	3.33 (8.61)	5.89 (13.00)	87	15.00 (22.78)	18.00 (25.09)	13.00 (21.12)	15.33 (23.00)
58	3.33 (10.40)	15.33 (23.03)	3.33 (10.49)	7.33 (14.64)	73	18.33 (25.33)	14.00 (21.93)	10.33 (18.62)	14.22 (21.96)	88	13.00 (21.06)	8.67 (17.07)	1.33 (5.42)	7.67 (14.52)
59	7.00 (15.27)	7.00 (15.27)	13.67 (21.67)	9.22 (17.40)	74	2.67 (9.36)	4.67 (12.41)	3.00 (9.97)	3.44 (10.58)	89	5.00 (12.46)	3.33 (10.40)	3.33 (10.40)	3.89 (11.09)
60	4.33 (11.76)	13.33 (21.37)	9.67 (18.09)	9.11 (17.07)	75	10.00 (18.39)	17.67 (24.83)	5.00 (12.87)	10.89 (18.70)	90	8.00 (16.42)	7.67 (16.06)	0.67 (2.71)	5.44 (11.73)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
91	6.00 (14.08)	4.33 (9.78)	0.00 (0.00)	3.44 (7.95)
92	15.00 (22.78)	15.67 (23.27)	2.33 (8.47)	11.00 (18.17)
93	16.67 (24.07)	19.67 (26.30)	20.67 (27.02)	19.00 (25.80)
94	0.00 (0.00)	19.33 (26.06)	19.33 (26.06)	12.89 (17.37)
95	4.33 (11.93)	2.67 (9.36)	0.00 (0.00)	2.33 (7.10)
96	2.00 (6.65)	8.67 (16.98)	3.67 (10.86)	4.78 (11.49)
97	3.33 (10.40)	15.33 (23.03)	3.33 (10.49)	7.33 (14.64)
98	0.00 (0.00)	19.00 (25.80)	6.33 (14.50)	8.44 (13.43)
99	3.33 (10.40)	15.00 (22.73)	11.00 (19.20)	9.78 (17.44)
Total Overall mean	10.30 (17.21)	9.46 (16.28)	8.59 (15.08)	9.45 (16.16)

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

Colony	(2.72)
Brood Cycle	(0.47)
Colony x Brood Cycle	(4.71)

Table 8. Percentage of brood cells only uncapped after 48 h of pricking in three alternate brood cycles*

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1	3.00 (9.88)	1.00 (4.62)	1.33 (3.84)	1.78 (6.12)	16	5.00 (12.92)	8.33 (16.77)	1.00 (5.74)	4.78 (11.81)	31	4.67 (11.56)	2.00 (8.13)	2.00 (8.13)	2.89 (8.63)
2	2.00 (8.13)	2.67 (9.36)	0.67 (2.71)	1.78 (6.73)	17	2.00 (8.13)	2.00 (8.13)	2.00 (8.13)	2.00 (6.52)	32	3.00 (9.97)	0.00 (0.00)	0.00 (0.00)	1.00 (3.32)
3	2.33 (8.47)	0.67 (2.71)	4.00 (11.47)	2.33 (7.55)	18	1.00 (3.32)	1.00 (4.62)	3.00 (9.54)	1.67 (5.83)	33	1.00 (3.32)	0.67 (2.71)	1.33 (6.53)	1.00 (4.19)
4	1.00 (4.62)	6.00 (13.91)	0.67 (2.71)	2.56 (7.08)	19	1.33 (5.24)	0.67 (2.71)	4.33 (11.89)	2.11 (6.61)	34	10.00 (18.41)	0.00 (0.00)	5.67 (13.25)	5.22 (10.55)
5	0.00 (0.00)	0.67 (2.71)	0.00 (0.00)	0.22 (0.90)	20	4.33 (11.66)	4.67 (12.13)	4.33 (11.74)	4.44 (11.84)	35	4.67 (12.41)	1.33 (5.42)	1.67 (6.03)	2.56 (7.95)
6	11.00 (19.36)	5.33 (13.26)	8.00 (16.42)	8.11 (16.35)	21	0.00 (0.00)	3.33 (10.40)	1.33 (5.42)	1.56 (5.27)	36	6.33 (14.39)	1.33 (3.84)	1.67 (6.03)	3.11 (8.09)
7	3.00 (9.97)	2.67 (7.63)	1.33 (3.84)	2.33 (7.15)	22	10.00 (18.43)	8.00 (16.11)	3.33 (10.34)	7.11 (14.96)	37	2.00 (8.13)	0.00 (0.00)	2.33 (8.56)	1.44 (5.56)
8	5.33 (13.33)	2.33 (5.42)	10.67 (19.23)	6.11 (13.70)	23	0.67 (3.82)	0.67 (3.82)	0.67 (3.82)	0.67 (3.82)	38	2.67 (9.36)	0.00 (0.00)	3.33 (10.06)	2.00 (6.47)
9	1.00 (4.62)	3.00 (8.05)	7.67 (16.06)	3.89 (9.58)	24	2.00 (7.94)	0.00 (0.00)	2.33 (8.74)	1.44 (5.56)	39	3.00 (9.97)	4.00 (11.37)	4.00 (11.28)	3.67 (10.87)
10	5.00 (12.92)	1.33 (6.53)	4.67 (12.35)	3.67 (10.60)	25	0.67 (2.71)	0.00 (0.00)	0.00 (0.00)	0.22 (0.90)	40	9.67 (17.05)	9.33 (17.30)	6.67 (14.95)	8.55 (16.43)
11	9.00 (17.45)	3.33 (8.57)	2.67 (9.26)	5.00 (11.76)	26	3.33 (10.50)	0.00 (0.00)	2.00 (6.65)	1.78 (5.71)	41	2.67 (7.43)	4.33 (11.89)	4.67 (12.28)	3.89 (10.53)
12	7.33 (12.91)	6.67 (14.79)	1.67 (6.03)	5.22 (11.24)	27	7.00 (11.69)	2.67 (7.69)	3.00 (9.60)	4.22 (9.66)	42	3.00 (8.05)	5.33 (13.29)	3.33 (9.96)	3.89 (10.43)
13	1.33 (3.84)	1.67 (6.03)	0.00 (0.00)	1.00 (3.29)	28	12.67 (20.65)	7.00 (17.93)	3.67 (10.95)	7.78 (15.64)	43	4.33 (9.66)	2.67 (9.08)	0.00 (0.00)	2.33 (6.25)
14	20.67 (26.98)	6.00 (14.14)	20.67 (26.98)	15.78 (22.70)	29	0.00 (0.00)	0.67 (3.82)	1.33 (5.42)	0.67 (3.08)	44	13.00 (21.13)	12.33 (20.55)	6.00 (14.04)	10.44 (18.57)
15	1.33 (2.71)	0.67 (2.71)	0.00 (0.00)	0.67 (2.18)	30	5.00 (12.92)	5.33 (13.26)	3.33 (10.34)	4.56 (12.17)	45	9.33 (18.43)	7.33 (15.65)	0.00 (0.00)	5.78 (11.36)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
46	7.67 (16.06)	2.33 (7.17)	2.33 (8.74)	4.11 (10.66)	61	2.00 (6.55)	1.67 (6.03)	2.00 (8.13)	1.89 (6.90)	76	3.00 (5.82)	3.00 (9.97)	10.67 (19.05)	5.56 (11.61)
47	3.67 (10.86)	3.00 (9.88)	1.00 (5.74)	2.56 (8.82)	62	4.00 (11.47)	2.33 (7.01)	3.33 (10.49)	3.22 (9.66)	77	2.00 (6.55)	2.00 (7.94)	6.00 (14.08)	3.33 (9.53)
48	4.33 (11.93)	1.33 (3.84)	1.33 (5.42)	2.32 (7.06)	63	4.00 (11.28)	4.00 (11.32)	0.67 (2.710)	2.89 (8.44)	78	2.00 (6.65)	8.67 (17.04)	11.33 (19.08)	7.33 (14.26)
49	7.33 (15.60)	8.67 (17.00)	4.67 (12.41)	6.89 (15.00)	64	2.00 (8.13)	1.33 (3.84)	1.00 (0.00)	1.44 (5.90)	79	10.00 (18.37)	4.33 (11.74)	4.00 (11.47)	6.11 (13.86)
50	2.00 (8.13)	3.67 (11.01)	5.00 (12.87)	3.56 (10.67)	65	8.67 (16.82)	3.67 (10.95)	8.67 (17.01)	7.00 (14.93)	80	2.00 (6.65)	2.00 (7.67)	8.00 (16.40)	4.00 (10.24)
51	2.67 (9.36)	9.00 (16.98)	5.33 (16.98)	6.89 (14.44)	66	8.33 (16.65)	2.00 (6.65)	0.67 (2.71)	3.67 (11.94)	81	0.00 (0.00)	6.67 (14.92)	6.00 (14.04)	4.22 (9.66)
52	2.33 (7.17)	5.33 (13.26)	0.00 (0.00)	2.56 (6.81)	67	11.67 (19.88)	2.00 (6.65)	1.33 (6.53)	5.00 (11.02)	82	0.00 (0.00)	0.00 (0.00)	1.33 (5.24)	0.44 (1.75)
53	2.00 (6.22)	2.00 (8.13)	2.67 (7.69)	2.22 (7.34)	68	8.00 (16.42)	6.00 (14.08)	4.00 (11.28)	6.00 (13.93)	83	1.33 (5.42)	1.33 (5.42)	2.33 (8.74)	1.66 (6.53)
54	4.00 (11.28)	4.00 (11.32)	0.67 (2.710)	2.89 (8.44)	69	6.00 (14.14)	1.00 (3.32)	2.00 (8.13)	3.00 (8.53)	84	0.00 (0.00)	0.67 (3.82)	1.33 (5.42)	0.67 (3.08)
55	2.00 (6.55)	1.33 (6.53)	2.00 (6.65)	1.78 (6.58)	70	0.00 (0.00)	4.67 (12.03)	3.67 (10.95)	2.78 (7.66)	85	0.00 (0.00)	4.00 (11.47)	6.67 (14.92)	3.56 (8.80)
56	3.67 (10.95)	2.67 (9.36)	1.33 (5.42)	2.56 (8.58)	71	8.67 (16.82)	5.67 (13.52)	6.33 (14.50)	6.89 (14.95)	86	4.67 (6.75)	0.67 (2.71)	3.67 (11.01)	2.78 (6.82)
57	2.67 (7.63)	1.33 (5.42)	2.00 (6.65)	2.00 (6.56)	72	1.33 (5.42)	1.33 (5.42)	2.33 (8.74)	1.66 (6.53)	87	7.00 (15.31)	7.67 (16.04)	7.67 (16.04)	7.45 (15.80)
58	2.33 (8.56)	5.67 (13.68)	1.00 (4.62)	3.00 (8.95)	73	8.67 (16.82)	3.67 (10.95)	8.67 (17.01)	7.00 (14.93)	88	1.00 (3.32)	0.00 (0.00)	1.00 (5.74)	0.67 (3.02)
59	2.67 (7.43)	4.33 (11.890)	4.67 (12.28)	3.89 (10.53)	74	3.00 (9.97)	4.00 (11.37)	4.00 (11.28)	3.67 (10.87)	89	1.33 (3.84)	1.33 (3.84)	0.00 (0.00)	.89 (2.56)
60	1.33 (5.24)	6.00 (14.07)	4.33 (11.99)	3.89 (10.43)	75	5.00 (12.64)	6.33 (14.50)	2.00 (6.55)	4.44 (11.23)	90	2.00 (0.00)	2.00 (8.13)	2.00 (6.65)	2.00 (7.63)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
91	2.00 (6.55)	2.00 (6.55)	4.67 (12.45)	2.89 (8.52)
92	7.00 (15.31)	2.00 (6.65)	0.00 (0.00)	3.00 (7.32)
93	9.00 (17.45)	2.00 (6.65)	8.67 (17.07)	6.56 (13.72)
94	3.00 (9.97)	7.00 (15.34)	7.00 (15.34)	5.67 (13.55)
95	1.67 (6.03)	0.00 (0.00)	0.67 (3.82)	0.78 (3.29)
96	3.00 (9.97)	2.67 (7.63)	1.33 (3.84)	2.33 (7.15)
97	2.33 (8.56)	5.67 (13.68)	1.00 (4.62)	3.00 (8.95)
98	3.00 (9.97)	2.00 (6.55)	2.33 (6.64)	2.44 (7.72)
99	2.33 (8.56)	4.00 (11.47)	8.67 (16.75)	5.00 (12.26)
Total mean	4.13 (9.80)	3.24 (8.59)	3.45 (8.87)	3.43 (8.65)

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

Colony	(3.57)
Brood Cycle	(0.62)
Colony x Brood Cycle	(6.19)

Table 9. Percentage of brood cells only uncapped after 72 h of pricking in three alternate brood cycles*

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1	0.00 (0.00)	0.67 (2.71)	0.00 (0.00)	0.22 (0.90)	16	2.00 (8.13)	4.67 (11.85)	0.00 (0.00)	2.22 (5.06)	31	3.33 (6.14)	0.00 (0.00)	5.67 (13.68)	4.78 (9.23)
2	1.00 (5.74)	2.67 (9.36)	1.00 (3.32)	1.56 (6.14)	17	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	32	0.33 (1.91)	0.00 (0.00)	0.00 (0.00)	0.11 (0.64)
3	1.67 (5.76)	0.67 (2.71)	1.33 (6.53)	1.22 (5.00)	18	0.00 (0.00)	1.00 (4.62)	2.67 (9.08)	1.22 (4.57)	33	0.67 (2.71)	0.67 (2.71)	1.33 (6.53)	0.89 (3.98)
4	0.33 (1.91)	2.33 (7.01)	0.67 (2.71)	1.11 (3.88)	19	0.00 (0.00)	1.00 (4.62)	3.00 (8.15)	1.33 (4.26)	34	5.00 (12.92)	0.00 (0.00)	2.67 (7.63)	2.56 (6.85)
5	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	20	3.00 (9.88)	2.00 (8.13)	2.67 (7.63)	2.56 (8.54)	35	1.33 (3.84)	0.67 (2.71)	0.67 (3.82)	0.89 (3.46)
6	11.00 (19.36)	2.67 (7.15)	3.00 (9.72)	5.22 (12.08)	21	0.00 (0.00)	3.33 (10.40)	1.33 (5.42)	1.56 (5.27)	36	3.33 (8.57)	0.67 (2.71)	0.33 (1.91)	1.44 (4.40)
7	3.00 (9.97)	1.67 (5.76)	1.33 (3.84)	2.00 (6.52)	22	10.00 (18.43)	3.33 (10.40)	0.00 (0.00)	4.44 (9.61)	37	2.00 (8.13)	0.00 (0.00)	0.00 (0.00)	0.67 (2.71)
8	2.33 (8.13)	2.33 (8.74)	1.00 (3.32)	1.78 (6.72)	23	0.67 (3.82)	0.67 (3.82)	0.67 (2.71)	0.67 (3.45)	38	1.67 (6.03)	0.00 (0.00)	2.00 (6.22)	1.22 (4.08)
9	1.00 (4.62)	1.00 (3.32)	1.00 (5.74)	1.00 (4.56)	24	2.00 (7.94)	0.00 (0.00)	1.67 (7.33)	1.22 (5.09)	39	3.00 (9.97)	2.33 (8.74)	3.33 (10.34)	2.89 (9.68)
10	5.00 (12.92)	1.33 (6.53)	3.00 (9.54)	3.11 (9.66)	25	0.33 (1.91)	0.00 (0.00)	0.00 (0.00)	0.11 (0.64)	40	5.67 (11.29)	0.00 (0.00)	6.00 (14.07)	3.89 (8.45)
11	5.00 (12.87)	2.33 (7.17)	0.00 (0.00)	2.44 (6.68)	26	3.33 (10.49)	0.00 (0.00)	0.00 (0.00)	1.11 (3.50)	41	2.00 (4.72)	3.33 (10.49)	0.67 (2.71)	2.00 (5.97)
12	6.00 (11.63)	4.67 (10.20)	1.00 (3.32)	3.89 (8.39)	27	5.33 (10.03)	1.33 (5.42)	0.33 (1.91)	2.33 (5.79)	42	0.67 (2.71)	3.67 (11.01)	0.00 (0.00)	1.44 (4.57)
13	1.33 (3.84)	0.00 (0.00)	0.00 (0.00)	0.44 (1.28)	28	7.67 (15.80)	7.33 (8.74)	2.67 (7.63)	4.22 (10.72)	43	3.67 (8.53)	2.67 (9.08)	0.00 (0.00)	2.11 (5.87)
14	15.33 (22.93)	4.33 (11.74)	15.33 (22.93)	11.67 (19.20)	29	0.67 (2.71)	0.00 (0.00)	1.33 (5.42)	0.67 (2.71)	44	2.00 (6.65)	5.00 (12.92)	3.33 (10.34)	3.44 (9.97)
15	0.00 (0.00)	0.33 (1.91)	0.00 (0.00)	0.11 (0.64)	30	5.00 (12.92)	4.67 (12.45)	1.67 (6.03)	3.78 (10.47)	45	4.00 (11.47)	3.33 (10.49)	0.00 (0.00)	2.44 (7.32)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
46	5.33 (13.29)	0.67 (9.75)	5.00 (9.35)	4.44 (10.80)	61	0.67 (2.71)	1.00 (4.62)	1.00 (3.32)	0.89 (3.55)	76	0.00 (0.00)	2.67 (9.36)	7.00 (15.25)	3.22 (8.20)
47	3.00 (9.88)	3.00 (9.88)	0.33 (1.91)	2.11 (7.22)	62	2.00 (6.65)	1.67 (6.03)	1.33 (3.84)	1.67 (5.51)	77	1.33 (3.84)	2.33 (8.56)	2.67 (9.26)	2.11 (7.22)
48	0.33 (1.91)	1.33 (3.84)	0.67 (2.71)	0.78 (2.82)	63	0.67 (2.71)	3.33 (8.61)	0.67 (2.71)	1.56 (4.68)	78	0.00 (0.00)	5.33 (12.93)	5.67 (12.46)	3.67 (8.46)
49	3.67 (10.76)	5.00 (12.49)	1.67 (6.03)	3.45 (9.76)	64	2.00 (8.13)	0.67 (2.71)	1.00 (5.73)	1.22 (5.53)	79	2.00 (6.65)	2.67 (7.43)	0.00 (0.00)	1.56 (4.69)
50	1.00 (0.00)	0.67 (2.71)	2.00 (7.94)	1.22 (5.46)	65	5.33 (13.10)	3.00 (9.97)	8.00 (16.13)	5.44 (13.07)	80	0.00 (0.00)	1.33 (3.84)	3.33 (10.34)	1.56 (4.73)
51	2.67 (9.36)	2.33 (7.17)	3.33 (7.17)	2.78 (7.90)	66	0.67 (2.71)	1.67 (6.03)	0.00 (0.00)	0.78 (2.91)	81	0.00 (0.00)	4.33 (11.99)	3.67 (11.01)	2.67 (7.67)
52	0.33 (1.91)	4.00 (11.47)	1.00 (3.32)	1.78 (5.57)	67	8.00 (16.37)	2.00 (6.65)	2.00 (8.13)	4.00 (10.38)	82	1.33 (5.42)	1.33 (5.42)	0.00 (0.00)	0.89 (3.61)
53	1.67 (4.31)	0.67 (2.71)	2.67 (7.69)	1.67 (4.90)	68	3.00 (9.97)	4.33 (11.89)	2.33 (7.01)	3.22 (9.63)	83	0.00 (0.00)	11.67 (19.90)	1.33 (5.42)	4.33 (8.44)
54	0.67 (2.71)	3.33 (8.61)	0.67 (2.71)	1.56 (4.68)	69	2.33 (8.74)	0.33 (1.91)	1.33 (5.42)	1.33 (5.36)	84	0.67 (2.71)	0.00 (0.00)	1.33 (5.42)	0.67 (2.71)
55	2.00 (6.55)	1.33 (6.53)	1.33 (3.84)	1.55 (5.64)	70	0.00 (0.00)	2.00 (4.72)	0.00 (0.00)	0.67 (1.57)	85	0.00 (0.00)	1.67 (6.03)	3.67 (10.95)	1.78 (5.66)
56	1.67 (6.03)	0.67 (2.71)	1.33 (5.42)	1.22 (4.72)	71	5.33 (13.10)	3.00 (9.54)	3.00 (9.88)	3.78 (10.84)	86	0.67 (2.71)	0.67 (2.71)	1.33 (5.42)	0.89 (3.61)
57	0.67 (2.71)	1.67 (7.33)	2.67 (9.36)	1.67 (6.46)	72	0.00 (0.00)	11.67 (19.90)	1.33 (5.42)	4.33 (8.44)	87	1.67 (7.15)	0.00 (0.00)	0.00 (0.00)	0.56 (2.38)
58	0.67 (3.82)	5.67 (13.68)	2.00 (7.94)	2.78 (8.48)	73	5.33 (13.10)	3.00 (9.97)	8.00 (16.13)	5.44 (13.07)	88	0.00 (0.00)	0.00 (0.00)	1.00 (5.74)	0.33 (1.91)
59	2.00 (4.72)	3.33 (10.49)	0.67 (2.71)	2.00 (5.97)	74	3.00 (9.97)	2.33 (8.74)	3.33 (10.34)	2.89 (9.68)	89	1.33 (3.84)	0.00 (0.00)	8.67 (0.00)	0.44 (1.28)
60	1.33 (5.24)	3.00 (8.15)	2.33 (8.13)	2.22 (7.17)	75	3.67 (10.95)	4.67 (12.13)	2.00 (6.55)	3.44 (9.88)	90	0.00 (0.00)	2.00 (8.13)	2.00 (6.65)	1.33 (4.93)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
91	2.00 (6.55)	2.00 (6.55)	0.67 (2.71)	1.56 (5.27)
92	1.67 (7.15)	2.00 (6.65)	0.00 (0.00)	1.22 (4.60)
93	3.67 (11.01)	2.33 (1.91)	0.00 (0.00)	1.33 (4.34)
94	3.00 (9.97)	7.00 (15.34)	7.00 (15.33)	5.67 (13.55)
95	1.67 (6.03)	0.00 (0.00)	0.00 (0.00)	0.56 (2.01)
96	3.00 (9.97)	1.67 (5.76)	1.33 (3.84)	2.00 (6.52)
97	0.67 (3.82)	5.67 (13.68)	2.00 (7.94)	2.78 (8.48)
98	3.00 (9.97)	2.67 (7.43)	2.00 (4.72)	2.56 (7.37)
99	0.67 (3.82)	1.33 (6.53)	6.67 (14.33)	2.89 (8.23)
Total mean	2.20 (6.42)	2.25 (6.52)	1.87 (5.49)	1.98 (5.73)

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

Colony (3.83)

Brood Cycle (0.67)

Colony x Brood Cycle (6.64)

Table 10. Percentage of brood cells remaining capped after 24 h of pricking in three alternate brood cycles*

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1	18.33 (25.34)	6.33 (14.50)	5.67 (13.72)	10.11 (17.86)	16	21.33 (27.50)	8.67 (17.04)	4.00 (9.45)	11.33 (18.00)	31	50.00 (44.98)	47.33 (43.45)	33.33 (35.25)	43.56 (41.22)
2	15.00 (22.78)	2.33 (8.74)	7.00 (15.27)	8.11 (15.60)	17	18.67 (25.58)	15.00 (22.77)	8.00 (16.42)	13.89 (21.59)	32	15.00 (22.75)	9.33 (17.75)	5.33 (13.16)	9.89 (17.89)
3	23.67 (29.09)	7.00 (15.27)	8.67 (17.07)	13.11 (20.48)	18	10.67 (17.89)	8.67 (17.07)	17.67 (24.81)	12.33 (19.92)	33	12.00 (20.23)	9.00 (17.45)	4.00 (11.53)	8.33 (16.40)
4	10.00 (18.43)	5.33 (13.26)	16.00 (23.56)	10.44 (18.42)	19	9.33 (17.78)	10.00 (18.38)	9.33 (17.70)	9.56 (17.95)	34	19.67 (40.88)	8.33 (16.59)	15.33 (23.03)	14.44 (21.98)
5	16.00 (23.52)	8.00 (16.42)	3.67 (10.86)	9.22 (16.93)	20	25.33 (30.06)	13.67 (21.61)	10.00 (18.33)	16.33 (23.33)	35	18.00 (25.09)	13.33 (23.86)	16.33 (23.80)	15.89 (23.41)
6.	13.00 (21.12)	10.33 (18.71)	6.67 (14.85)	10.00 (18.23)	21	8.33 (16.68)	5.33 (13.16)	4.33 (11.66)	6.00 (13.84)	36	15.00 (22.75)	10.33 (18.61)	9.67 (18.10)	11.67 (19.82)
7	11.67 (19.88)	13.33 (21.41)	12.67 (20.80)	12.56 (20.69)	22	2.33 (8.47)	16.67 (24.08)	14.33 (22.20)	11.11 (18.25)	37	19.00 (26.75)	10.67 (18.78)	8.67 (18.09)	12.78 (20.54)
8	16.33 (23.82)	2.33 (8.47)	8.67 (17.09)	9.11 (16.46)	23	9.00 (17.45)	9.00 (17.45)	9.33 (17.75)	9.11 (17.55)	38	11.00 (19.20)	6.67 (14.77)	7.00 (15.31)	8.22 (16.43)
9	14.00 (21.94)	12.33 (19.95)	15.67 (23.28)	14.00 (21.73)	24	15.67 (23.30)	13.00 (21.13)	9.33 (17.75)	12.67 (20.72)	39	9.33 (17.78)	15.67 (23.30)	14.00 (21.96)	13.00 (21.01)
10	14.00 (21.96)	4.67 (12.12)	11.67 (19.95)	10.11 (18.01)	25	22.00 (27.95)	7.33 (15.65)	9.00 (17.29)	12.78 (20.30)	40	59.33 (50.37)	42.00 (40.38)	17.33 (24.56)	39.56 (38.44)
11	14.67 (22.51)	14.00 (21.89)	7.00 (15.34)	11.89 (19.91)	26	15.00 (22.77)	8.33 (16.68)	10.67 (18.98)	11.33 (19.47)	41	16.00 (23.57)	4.67 (12.28)	11.00 (19.35)	10.56 (18.40)
12	0.00 (0.00)	11.67 (19.75)	7.00 (15.25)	6.22 (11.67)	27	16.00 (23.55)	18.33 (25.34)	5.67 (13.72)	13.33 (20.87)	42	9.00 (17.45)	6.67 (14.79)	11.67 (19.94)	9.11 (17.39)
13	5.00 (12.46)	17.67 (24.84)	13.00 (21.12)	11.89 (19.47)	28	6.00 (14.17)	20.00 (26.55)	19.00 (25.83)	15.00 (22.18)	43	12.00 (20.26)	12.67 (20.83)	15.00 (22.72)	13.22 (21.27)
14	8.33 (16.68)	5.33 (13.29)	8.33 (16.68)	7.33 (15.55)	29	22.33 (28.17)	19.00 (25.82)	10.67 (18.98)	17.33 (24.32)	44	56.00 (48.43)	17.00 (11.89)	24.00 (29.31)	32.33 (34.02)
15	7.67 (15.71)	8.67 (17.07)	6.67 (14.89)	7.67 (15.89)	30	16.67 (24.08)	9.00 (17.29)	11.00 (19.31)	12.22 (20.23)	45	29.67 (32.97)	11.67 (19.92)	21.33 (27.48)	20.89 (24.16)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
46	13.00 (21.02)	18.67 (25.58)	12.33 (20.51)	14.67 (22.37)	61	15.33 (23.03)	12.00 (20.23)	14.33 (22.17)	13.89 (21.81)	76	17.00 (24.34)	27.00 (31.29)	11.67 (19.90)	18.56 (25.18)
47	17.00 (32.32)	2.67 (8.93)	22.00 (27.96)	13.89 (20.40)	62	25.67 (30.39)	12.67 (20.84)	5.67 (13.72)	14.67 (21.65)	77	10.33 (18.62)	5.33 (13.33)	28.67 (32.36)	14.78 (21.44)
48	13.00 (34.70)	9.33 (17.68)	7.33 (15.65)	9.89 (18.15)	63	28.00 (31.92)	6.67 (21.20)	9.67 (17.99)	14.78 (21.61)	78	23.00 (28.64)	5.00 (12.87)	8.00 (916.36)	12.00 (19.29)
49	17.00 (24.34)	12.00 (20.18)	11.00 (19.35)	13.33 (21.29)	64	15.67 (23.30)	3.33 (10.49)	14.33 (22.24)	11.11 (18.68)	79	11.67 (19.96)	4.33 (24.33)	16.00 (23.55)	10.67 (18.47)
50	18.67 (25.55)	13.00 (21.10)	14.00 (21.96)	15.22 (22.87)	65	11.67 (19.89)	4.33 (11.99)	10.67 (19.05)	8.89 (16.98)	80	23.00 (28.64)	6.00 (14.14)	24.67 (28.88)	17.89 (23.89)
51	19.67 (26.23)	7.33 (15.59)	7.33 (15.59)	11.44 (19.14)	66	17.67 (24.84)	8.33 (16.77)	9.33 (17.70)	11.78 (19.77)	81	14.00 (21.70)	10.00 (18.37)	17.00 (24.32)	13.67 (21.46)
52	30.00 (27.96)	15.00 (22.77)	14.00 (21.94)	17.00 (24.22)	67	15.00 (22.78)	15.67 (23.25)	9.33 (17.70)	13.33 (21.25)	82	20.00 (26.50)	18.67 (25.59)	13.67 (21.64)	17.44 (24.57)
53	14.00 (21.96)	27.67 (31.72)	9.33 (17.78)	17.00 (23.82)	68	10.00 (18.43)	15.33 (23.03)	21.33 (27.49)	15.56 (22.98)	83	14.00 (21.94)	12.33 (20.46)	5.00 (12.80)	10.44 (18.40)
54	11.00 (19.35)	7.33 (15.59)	12.00 (20.23)	10.11 (18.39)	69	9.00 (17.43)	9.67 (18.07)	19.00 (25.36)	12.56 (20.29)	84	22.33 (28.17)	19.00 (25.82)	10.67 (18.98)	17.33 (24.32)
55	28.67 (32.35)	10.33 (18.72)	17.00 (24.32)	18.67 (25.13)	70	3.67 (11.01)	16.67 (24.07)	23.33 (28.87)	14.56 (21.32)	85	12.33 (20.55)	13.33 (21.41)	29.33 (32.76)	18.33 (24.91)
56	9.00 (17.400)	14.67 (22.51)	8.00 (16.20)	10.56 (18.70)	71	13.00 (21.10)	16.33 (23.82)	11.67 (19.89)	13.67 (21.60)	86	10.00 (18.43)	9.67 (18.10)	19.00 (25.82)	12.89 (20.78)
57	29.00 (32.54)	13.33 (21.41)	11.33 (19.65)	17.89 (24.53)	72	3.67 (11.01)	13.00 (21.02)	11.33 (19.65)	9.33 (17.23)	87	12.00 (20.25)	12.00 (20.25)	22.00 (20.25)	12.00 (20.25)
58	16.00 (23.57)	4.33 (11.74)	13.67 (21.67)	11.33 (18.99)	73	13.00 (21.10)	5.33 (13.33)	4.67 (12.41)	7.67 (15.62)	88	9.00 (17.45)	13.67 (21.68)	6.67 (14.95)	9.78 (18.02)
59	16.00 (23.57)	4.67 (12.28)	11.00 (19.35)	10.56 (18.40)	74	14.00 (21.70)	13.00 (21.12)	11.33 (19.49)	12.78 (20.77)	89	10.00 (18.43)	15.67 (23.30)	15.67 (23.30)	13.78 (21.68)
60	6.00 (14.08)	13.00 (21.12)	9.33 (17.76)	9.44 (17.65)	75	16.00 (23.55)	6.67 (14.89)	11.67 (19.82)	11.44 (19.42)	90	9.00 (17.45)	4.33 (11.99)	12.00 (20.26)	8.44 (16.57)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
91	4.67 (12.41)	5.00 (12.87)	25.33 (30.14)	11.67 (18.48)
92	12.00 (20.25)	8.00 (16.42)	20.67 (27.03)	13.56 (21.23)
93	35.00 (16.77)	5.67 (13.75)	16.00 (23.52)	18.89 (18.01)
94	13.33 (21.36)	4.67 (12.45)	4.67 (12.45)	7.56 (15.42)
95	15.33 (22.98)	8.67 (17.00)	9.00 (17.43)	11.00 (19.14)
96	11.67 (19.88)	13.33 (21.41)	12.67 (20.80)	12.56 (20.69)
97	16.00 (23.57)	4.33 (11.74)	13.67 (21.67)	11.33 (18.99)
98	13.33 (21.36)	6.67 (14.95)	4.00 (11.47)	8.00 (15.93)
99	13.00 (23.57)	8.00 (16.34)	16.33 (23.81)	12.44 (21.24)
Total mean	15.28 (22.34)	11.28 (18.85)	12.31 (19.92)	15.20 (20.24)

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

Colony	(1.94)
Brood Cycle	(0.34)
Colony x Brood Cycle	(3.36)

Table 11. Percentage of brood cells remaining capped after 48 h of pricking in three alternate brood cycles*

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1	18.33 (25.34)	5.00 (12.80)	5.00 (12.92)	9.44 (17.02)	16	19.00 (25.83)	8.67 (17.04)	4.00 (9.45)	10.56 (17.44)	31	31.00 (33.68)	34.00 (35.64)	28.00 (31.93)	31.00 (33.75)
2	13.00 (21.12)	2.00 (8.13)	4.33 (11.36)	6.44 (13.54)	17	8.00 (16.42)	8.00 (16.42)	8.00 (16.42)	8.00 (16.42)	32	13.67 (21.63)	7.67 (16.02)	5.33 (13.16)	8.89 (16.94)
3	9.33 (17.71)	6.33 (14.56)	8.67 (17.07)	8.11 (16.45)	18	3.67 (10.95)	7.33 (15.65)	13.33 (21.39)	8.11 (16.00)	33	7.67 (16.06)	8.33 (16.75)	4.00 (11.53)	6.67 (14.78)
4	10.00 (18.43)	5.33 (13.26)	12.00 (20.25)	9.11 (17.31)	19	5.33 (13.16)	8.67 (17.11)	7.67 (15.98)	7.22 (15.42)	34	15.67 (23.30)	6.33 (14.50)	12.33 (20.53)	11.44 (19.44)
5	10.67 (18.98)	6.33 (14.43)	3.33 (10.40)	6.78 (14.60)	20	8.67 (16.98)	6.67 (14.85)	6.00 (13.97)	7.11 (15.26)	35	15.00 (22.78)	10.00 (20.82)	10.00 (18.37)	11.67 (20.66)
6	9.67 (18.10)	10.33 (17.91)	4.67 (12.28)	8.22 (16.36)	21	6.33 (14.56)	3.00 (8.05)	3.67 (10.53)	4.33 (11.05)	36	9.67 (17.82)	8.33 (16.46)	5.33 (13.26)	7.78 (15.85)
7	10.67 (19.04)	10.33 (18.68)	11.00 (19.21)	10.67 (18.98)	22	2.33 (8.47)	15.67 (23.30)	10.33 (18.68)	9.44 (16.81)	37	15.00 (22.78)	6.00 (13.97)	7.00 (15.25)	9.33 (17.33)
8	16.33 (23.82)	2.33 (8.47)	6.67 (14.92)	8.44 (15.74)	23	3.67 (10.95)	3.67 (10.95)	9.33 (17.75)	5.56 (13.22)	38	7.00 (15.17)	5.33 (13.29)	5.33 (13.26)	5.89 (13.91)
9	5.33 (13.26)	6.00 (14.04)	10.00 (18.43)	7.11 (15.24)	24	13.00 (21.12)	11.00 (19.36)	6.33 (14.56)	10.11 (18.35)	39	9.33 (17.78)	8.67 (16.88)	10.33 (18.71)	9.44 (17.79)
10	11.00 (19.36)	3.67 (10.95)	6.67 (14.77)	7.11 (15.03)	25	7.00 (15.14)	6.67 (14.95)	6.67 (14.95)	6.78 (15.01)	40	24.67 (29.70)	20.33 (26.79)	14.33 (22.24)	19.78 (26.24)
11	13.00 (21.13)	5.67 (13.62)	7.00 (15.34)	8.56 (16.70)	26	13.00 (21.12)	5.00 (12.80)	8.33 (16.49)	8.78 (16.80)	41	11.00 (19.15)	3.00 (9.72)	7.67 (15.94)	7.22 (14.94)
12	3.00 (5.82)	8.67 (14.07)	4.33 (11.93)	5.33 (10.61)	27	8.00 (16.23)	11.67 (19.96)	5.67 (13.72)	8.44 (16.64)	42	7.33 (15.19)	3.33 (8.57)	9.00 (17.43)	6.56 (13.73)
13	4.67 (11.85)	10.67 (18.89)	11.00 (19.35)	8.78 (16.70)	28	2.67 (9.36)	20.00 (26.55)	14.67 (22.37)	12.44 (19.43)	43	18.67 (24.51)	2.67 (9.36)	8.00 (16.42)	9.78 (16.76)
14	5.00 (12.92)	2.67 (9.26)	5.00 (12.92)	4.22 (11.70)	29	16.00 (23.51)	11.67 (19.75)	9.33 (17.76)	12.33 (20.34)	44	14.00 (26.66)	13.67 (9.88)	22.00 (27.96)	16.56 (23.86)
15	6.67 (14.27)	7.67 (16.06)	2.33 (8.47)	5.56 (12.93)	30	15.33 (23.03)	7.33 (14.98)	6.33 (14.29)	9.67 (17.43)	45	15.00 (26.14)	8.00 (16.29)	15.33 (23.03)	12.78 (20.70)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
46	8.00 (16.11)	8.00 (16.07)	9.67 (18.04)	8.56 (16.74)	61	9.00 (17.38)	7.67 (16.06)	10.00 (18.38)	8.89 (17.28)	76	11.33 (19.65)	7.00 (15.27)	9.00 (17.45)	9.11 (17.46)
47	13.33 (21.35)	2.67 (8.93)	18.00 (25.09)	11.33 (18.46)	62	12.00 (20.22)	6.00 (13.86)	5.00 (12.92)	7.67 (15.67)	77	5.33 (13.16)	2.67 (9.36)	11.33 (19.66)	6.44 (14.06)
48	10.67 (20.92)	7.00 (15.34)	5.33 (13.29)	7.67 (15.89)	63	10.00 (18.38)	4.67 (12.45)	7.33 (15.70)	7.33 (15.51)	78	16.67 (24.07)	3.67 (10.95)	6.00 (11.63)	8.78 (15.55)
49	11.33 (19.61)	7.00 (15.23)	8.67 (17.11)	9.00 (17.32)	64	15.67 (23.30)	3.33 (10.49)	14.33 (22.24)	11.11 (18.68)	79	10.67 (19.04)	3.00 (9.88)	14.33 (22.19)	9.33 (17.04)
50	16.67 (24.07)	8.00 (16.37)	14.00 (34.64)	12.89 (20.80)	65	10.33 (18.72)	5.33 (13.33)	1.00 (3.32)	5.56 (11.79)	80	14.00 (24.07)	5.33 (13.33)	6.00 (14.14)	8.44 (17.18)
51	13.67 (21.59)	4.67 (12.41)	22.67 (28.42)	13.67 (20.81)	66	17.00 (24.33)	6.00 (13.86)	6.33 (14.56)	9.78 (17.59)	81	8.67 (16.95)	6.00 (14.07)	11.00 (19.35)	8.56 (16.79)
52	19.33 (26.02)	5.33 (12.98)	12.67 (20.84)	12.44 (19.95)	67	10.00 (18.43)	9.00 (17.40)	5.33 (13.16)	8.11 (16.33)	82	17.00 (24.34)	17.00 (24.34)	13.00 (21.09)	15.67 (23.26)
53	14.00 (21.89)	14.33 (22.17)	7.33 (15.59)	11.89 (19.88)	68	10.00 (18.43)	5.67 (13.68)	11.67 (19.82)	9.11 (17.31)	83	11.67 (19.90)	11.67 (19.90)	4.67 (12.41)	9.33 (17.40)
54	10.00 (18.38)	4.67 (12.45)	7.33 (15.70)	7.33 (15.51)	69	9.00 (17.43)	7.67 (16.04)	7.00 (15.34)	7.89 (16.27)	84	16.00 (23.51)	11.67 (19.75)	9.33 (17.76)	12.33 (20.34)
55	8.67 (17.09)	10.33 (18.72)	10.33 (18.74)	9.78 (18.18)	70	3.67 (11.01)	13.67 (21.65)	16.00 (23.57)	11.11 (18.74)	85	11.67 (19.96)	10.67 (19.98)	16.33 (23.80)	12.89 (20.91)
56	7.33 (15.67)	14.67 (22.51)	6.00 (13.40)	9.33 (17.19)	71	10.33 (18.72)	13.67 (21.64)	8.67 (17.11)	10.89 (19.16)	86	9.00 (17.43)	9.00 (17.43)	12.67 (20.83)	10.22 (18.57)
57	16.33 (23.77)	6.67 (14.95)	8.67 (17.09)	10.56 (18.60)	72	11.67 (19.90)	11.67 (19.90)	4.67 (12.41)	9.33 (17.40)	87	11.00 (19.31)	12.00 (20.25)	12.00 (20.25)	11.67 (19.94)
58	8.67 (16.74)	2.33 (8.56)	9.00 (17.43)	6.67 (14.24)	73	10.33 (18.72)	5.33 (13.33)	1.00 (3.32)	5.56 (11.79)	88	9.00 (17.45)	11.67 (19.96)	4.00 (11.53)	8.22 (16.31)
59	11.00 (19.15)	3.00 (9.72)	7.67 (15.94)	7.22 (14.94)	74	9.33 (17.78)	8.67 (16.88)	10.33 (18.71)	9.44 (17.79)	89	10.33 (18.34)	13.67 (21.68)	23.33 (21.68)	15.78 (20.56)
60	5.00 (12.92)	6.33 (14.39)	8.67 (17.01)	6.67 (14.77)	75	12.67 (20.65)	5.67 (13.72)	5.00 (12.49)	7.78 (15.62)	90	9.00 (17.45)	3.33 (10.49)	8.00 (16.42)	6.78 (14.79)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
91	4.00 (11.53)	4.00 (11.53)	12.67 (20.84)	6.89 (14.63)
92	11.00 (23.01)	6.00 (14.04)	15.33 (23.03)	10.78 (18.79)
93	8.33 (16.77)	3.67 (11.01)	13.67 (21.67)	8.56 (16.48)
94	7.33 (15.65)	3.00 (9.97)	3.00 (9.97)	4.44 (11.86)
96	10.67 (19.04)	10.33 (18.68)	11.00 (19.21)	10.67 (18.98)
97	8.67 (16.74)	2.33 (8.56)	9.00 (17.43)	6.67 (14.24)
98	7.33 (15.65)	4.33 (11.99)	4.00 (11.47)	5.22 (13.04)
99	8.67 (16.74)	8.00 (16.34)	13.00 (21.01)	9.89 (18.03)
Mean	10.80 (18.67)	7.77 (15.48)	8.99 (16.82)	9.19 (16.99)

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

LSD ($p=0.05$) for:

Colony	(2.47)
Brood Cycle	(0.43)
Colony x Brood Cycle	(4.28)

Table 12. Percentage of brood cells remaining capped after 72 h of pricking in three alternate brood cycles*

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1	18.33 (25.34)	5.00 (12.80)	5.00 (12.92)	9.44 (17.02)	16	19.00 (25.83)	7.67 (16.06)	4.00 (9.45)	10.22 (17.11)	31	26.00 (30.40)	22.67 (28.39)	20.33 (26.79)	23.00 (28.53)
2	13.00 (21.12)	2.00 (8.13)	4.00 (10.94)	6.33 (13.40)	17	8.00 (16.42)	8.00 (16.42)	8.00 (16.42)	8.00 (16.42)	32	13.67 (21.63)	7.33 (15.67)	5.33 (13.16)	8.78 (16.82)
3	9.33 (17.71)	6.33 (14.56)	8.67 (17.07)	8.11 (16.45)	18	3.67 (10.95)	6.67 (14.95)	11.67 (19.75)	7.33 (15.22)	33	7.00 (15.25)	7.67 (16.04)	4.00 (11.53)	6.22 (14.28)
4	10.00 (18.43)	5.33 (13.26)	12.00 (20.25)	9.11 (17.31)	19	4.00 (11.47)	8.00 (16.36)	7.33 (15.56)	6.44 (14.46)	34	15.67 (23.3)	6.33 (14.50)	10.00 (18.38)	10.67 (18.73)
5	9.67 (18.05)	6.33 (14.43)	3.33 (10.40)	6.44 (14.29)	20	7.67 (15.87)	6.67 (14.85)	5.67 (13.62)	6.67 (14.78)	35	15.00 (22.78)	12.67 (20.82)	6.67 (14.89)	11.44 (19.50)
6	9.67 (18.10)	10.33 (18.71)	4.67 (12.28)	8.22 (16.36)	21	6.33 (14.56)	3.00 (8.05)	3.67 (10.53)	4.33 (11.05)	36	9.00 (17.01)	8.33 (16.46)	5.33 (13.26)	7.56 (15.58)
7	10.67 (19.04)	7.33 (15.41)	6.33 (14.27)	8.11 (16.24)	22	2.33 (8.47)	15.67 (23.30)	10.00 (18.33)	9.33 (16.70)	37	15.00 (22.78)	6.00 (13.97)	7.00 (15.25)	9.33 (17.33)
8	16.33 (23.82)	2.33 (8.47)	6.00 (14.04)	8.22 (15.44)	23	3.67 (10.95)	3.67 (10.95)	6.00 (14.08)	4.44 (12.00)	38	4.67 (12.28)	5.33 (13.29)	5.33 (13.26)	5.11 (12.94)
9	4.67 (12.28)	5.33 (13.29)	5.00 (12.92)	5.00 (12.83)	24	13.00 (21.12)	11.00 (19.36)	4.67 (12.41)	9.56 (17.63)	39	9.33 (17.78)	8.00 (16.23)	8.33 (16.53)	8.56 (16.85)
10	11.00 (19.36)	3.00 (9.97)	5.67 (13.51)	6.56 (14.28)	25	5.33 (12.50)	5.33 (13.33)	6.67 (14.95)	5.78 (13.59)	40	21.00 (27.18)	20.33 (26.79)	10.67 (19.04)	17.33 (24.33)
11	13.00 (21.12)	5.67 (13.62)	7.00 (15.34)	8.56 (16.70)	26	13.00 (21.12)	8.00 (15.54)	8.33 (16.49)	9.78 (17.72)	41	10.33 (18.44)	3.00 (9.72)	6.33 (14.43)	6.56 (14.20)
12	2.67 (5.47)	8.00 (13.46)	4.33 (11.93)	5.00 (10.29)	27	8.00 (16.23)	11.67 (19.96)	5.67 (13.72)	8.44 (16.64)	42	7.33 (15.19)	4.00 (11.28)	9.00 (17.43)	6.78 (14.63)
13	4.67 (11.85)	9.00 (17.16)	11.00 (19.35)	8.22 (16.12)	28	1.33 (5.24)	20.00 (26.55)	14.67 (22.37)	12.00 (18.05)	43	15.00 (22.22)	2.67 (9.36)	8.00 (16.42)	8.56 (16.00)
14	2.00 (7.94)	2.33 (8.74)	2.00 (7.94)	2.11 (8.21)	29	13.00 (21.06)	11.67 (19.75)	6.67 (14.92)	10.44 (18.58)	44	11.67 (15.19)	13.67 (11.28)	16.67 (17.43)	14.00 (21.90)
15	6.00 (13.64)	7.67 (16.06)	2.33 (8.47)	5.33 (12.72)	30	15.33 (23.03)	7.33 (14.98)	5.33 (13.26)	9.33 (21.56)	45	15.00 (22.78)	7.00 (15.31)	15.33 (23.03)	12.44 (20.37)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
46	7.33 (15.41)	8.00 (16.07)	6.33 (14.43)	7.22 (15.30)	61	7.33 (15.48)	7.00 (15.31)	8.00 (16.36)	7.44 (15.72)	76	11.33 (19.65)	6.33 (14.56)	9.00 (17.45)	8.89 (17.22)
47	10.00 (18.08)	2.67 (8.93)	16.00 (23.56)	9.56 (16.86)	62	12.00 (20.22)	4.67 (12.28)	5.00 (12.92)	7.22 (15.14)	77	4.67 (12.35)	2.67 (9.36)	8.33 (16.77)	5.22 (12.82)
48	10.00 (18.43)	7.00 (15.34)	5.33 (13.29)	7.44 (15.69)	63	10.00 (18.38)	4.67 (12.45)	7.33 (15.70)	7.33 (15.51)	78	16.67 (24.07)	3.67 (10.95)	6.00 (11.63)	8.78 (15.55)
49	10.33 (18.68)	7.00 (15.23)	8.67 (17.11)	8.67 (17.01)	64	15.67 (23.30)	3.33 (10.49)	14.33 (22.24)	11.11 (18.68)	79	10.67 (19.04)	13.67 (9.88)	14.33 (22.19)	9.33 (21.91)
50	8.00 (22.49)	8.00 (16.37)	14.00 (21.96)	10.00 (20.27)	65	10.33 (18.72)	5.33 (13.33)	1.00 (3.32)	5.56 (11.79)	80	16.67 (24.07)	5.33 (13.33)	13.33 (21.13)	11.78 (19.51)
51	12.00 (20.14)	2.33 (7.18)	4.67 (12.41)	6.33 (13.24)	66	17.00 (24.33)	5.33 (12.98)	6.33 (14.56)	9.56 (17.29)	81	8.67 (16.95)	6.00 (14.07)	11.00 (19.35)	8.56 (16.79)
52	13.00 (20.97)	5.33 (12.98)	7.33 (15.67)	8.56 (16.54)	67	10.00 (18.43)	7.67 (16.06)	4.67 (12.12)	7.44 (15.54)	82	15.67 (23.30)	15.67 (23.30)	7.00 (15.25)	12.78 (20.62)
53	13.00 (21.10)	12.33 (20.46)	6.67 (14.92)	10.67 (18.83)	68	10.00 (18.43)	4.67 (12.13)	8.67 (17.09)	7.78 (15.88)	83	11.67 (19.90)	0.67 (2.71)	5.33 (13.16)	5.89 (11.92)
54	10.00 (18.38)	4.67 (12.45)	7.33 (15.70)	7.33 (15.51)	69	9.00 (17.43)	7.00 (15.34)	5.67 (13.72)	7.22 (16.70)	84	13.00 (21.06)	11.67 (19.75)	6.67 (14.92)	10.44 (18.58)
55	8.00 (16.42)	10.33 (18.72)	8.33 (16.59)	8.89 (17.24)	70	3.67 (11.01)	10.67 (18.97)	9.67 (17.41)	8.00 (15.80)	85	11.67 (19.96)	9.67 (17.81)	12.33 (20.55)	11.22 (19.44)
56	7.33 (15.67)	14.67 (22.51)	5.00 (12.46)	9.00 (16.88)	71	10.33 (18.72)	12.00 (20.16)	6.67 (14.95)	9.67 (17.95)	86	9.00 (17.43)	9.00 (17.43)	12.67 (20.83)	10.22 (18.57)
57	13.67 (21.68)	5.67 (13.55)	7.67 (16.06)	9.00 (17.09)	72	11.67 (19.90)	0.67 (2.71)	5.33 (13.16)	5.89 (11.92)	87	10.33 (18.72)	12.00 (20.25)	12.00 (20.25)	11.44 (19.74)
58	8.00 (16.13)	2.33 (8.56)	6.00 (14.04)	5.44 (12.91)	73	10.33 (18.72)	5.33 (13.33)	1.00 (3.32)	5.56 (11.79)	88	9.00 (17.45)	11.67 (19.96)	4.00 (11.53)	8.22 (16.31)
59	10.33 (18.44)	3.00 (9.72)	6.33 (14.43)	6.56 (14.20)	74	9.33 (17.78)	8.00 (16.23)	8.33 (16.53)	8.56 (16.85)	89	10.33 (18.34)	13.67 (21.68)	13.67 (21.68)	12.56 (20.56)
60	5.00 (12.92)	5.67 (13.68)	8.67 (17.01)	6.44 (14.54)	75	10.33 (18.50)	5.67 (13.68)	3.67 (10.86)	6.56 (14.35)	90	9.00 (17.45)	3.33 (10.49)	8.00 (16.42)	6.78 (14.79)

Col. No.	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
91	4.00 (11.53)	4.00 (11.53)	11.67 (19.90)	6.56 (14.32)
92	10.33 (18.72)	6.00 (14.04)	14.33 (22.24)	10.22 (18.33)
93	8.33 (16.77)	3.67 (11.01)	13.67 (21.67)	8.56 (16.48)
94	7.33 (15.65)	3.00 (9.97)	3.00 (9.97)	4.44 (11.86)
95	4.67 (11.56)	6.67 (14.79)	9.00 (14.56)	6.78 (13.64)
96	10.67 (19.04)	7.33 (15.41)	6.33 (14.27)	8.11 (16.24)
97	8.00 (16.13)	2.33 (8.56)	6.00 (14.04)	5.44 (12.91)
98	7.33 (15.65)	4.33 (11.99)	4.00 (11.47)	5.22 (13.04)
99	8.00 (16.13)	8.00 (16.34)	13.00 (21.01)	9.67 (17.83)
Mean	10.05 (18.04)	7.10 (14.83)	7.76 (15.57)	8.30 (16.14)

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

Colony	(2.56)
Brood Cycle	(0.45)
Colony x Brood Cycle	(4.43)

4.1.4 Grouping of the screened 100 colonies into different classes of hygienic behaviour

Out of the 100 colonies screened for hygienic behaviour, 45 colonies were hygienic; 55 were non-hygienic out of which 42 were intermediary (71-80% emptying of pricked brood cells), and 13 were the least hygienic colonies (<71% emptying of pricked brood cells). The different groups w.r.t. hygienic response i.e. the expression of the hygienic traits displayed, in terms of the percentage of emptying of the pricked brood within the various time intervals of pricking, for the various colonies, were 41-50, 61-70, 71-80, 81-90 and 91-100 per cent, respectively. After 24 h of pricking, one colony fell in the group of 91-100 per cent hygienic, 44 colonies in 81-90 per cent hygienic group, 42 were in 71-80 per cent group, 10 in 61-70 per cent group and three into 41-50 per cent hygienic group. After 48 h, the scenario of the number of colonies in various groups changed significantly. Ninety five colonies i.e. (83 and 12), were in the two different groups, i.e. 81-90 and 91-100 per cent hygienic, respectively; four colonies belonged to 71-80 per cent group and one into 61-70 per cent hygienic group. At 72 hours, however the maximum of the colonies depicted to have expressed hygienic response; 64 colonies grouped in 81-90 and 34 in 91-100 per cent hygienic group. Only two colonies were present in intermediary group 71-80 per cent (Table 13, Fig. 1). In the 45 hygienic colonies, recorded after 24 h, the mean pin killed brood removal was 83.65 per cent (range: 80.22-91.0 %), whereas it was 73.05 per cent (range: 46.78-79.56%) in the remaining 55 colonies. After 48 h, 94 colonies manifested mean of 87.27 per cent (range: 81.33-94.11%) emptied brood cells whereas after 72 h, the corresponding value was 89.77 per cent (range: 82.55-94.89%) expressed by 98 colonies.

4.1.5 Selection of colonies from the colonies screened out

Based on the hygienic response of the evaluated 100 colonies expressed within the first 24 h of the brood pricking, 10 colonies (7 the most hygienic and 3 the most non-hygienic) were primarily selected for further evaluation and molecular characterization of hygienic behaviour.

Based on the confirmatory field tests conducted, in the seven hygienic colonies selected, the mean pin killed brood cells emptied (hygienic behaviour) ranged between 91.00-86.44 per cent, and for the three non-hygienic colonies the hygienic values were 46.78, 46.89 and 47.33 per cent, respectively. These seven selected hygienic colonies were designated as 1H to 7H and non-hygienic colonies as 1NH to 3NH. The seven hygienic colonies had expressed statistically significant differences in expression of hygienic response when compared with the three non-hygienic colonies. Within the seven hygienic colonies, colony 3H (91.00%) was statistically at par with colony 1H (89.33%), whereas colony 2H was statistically at par with colony colonies 4H, 6H, 7H and 5H, expressing, 88.33, 88.00, 87.11 and 86.44 per cent hygienic behaviour, respectively. The three non-hygienic colonies were statistically at par with each other (Table 14, Fig. 2).

Table 13. Grouping of the screened 100 colonies based on the mean percentage of hygienic behaviour (brood removal following pin killing)

Classes of mean per cent hygienic behaviour	No. of colonies		
	24h	48h	72h
41-50	3	0	0
61- 70	10	1	0
71-80	42	4	2
81-90	44	83	64
91-100	1	12	34

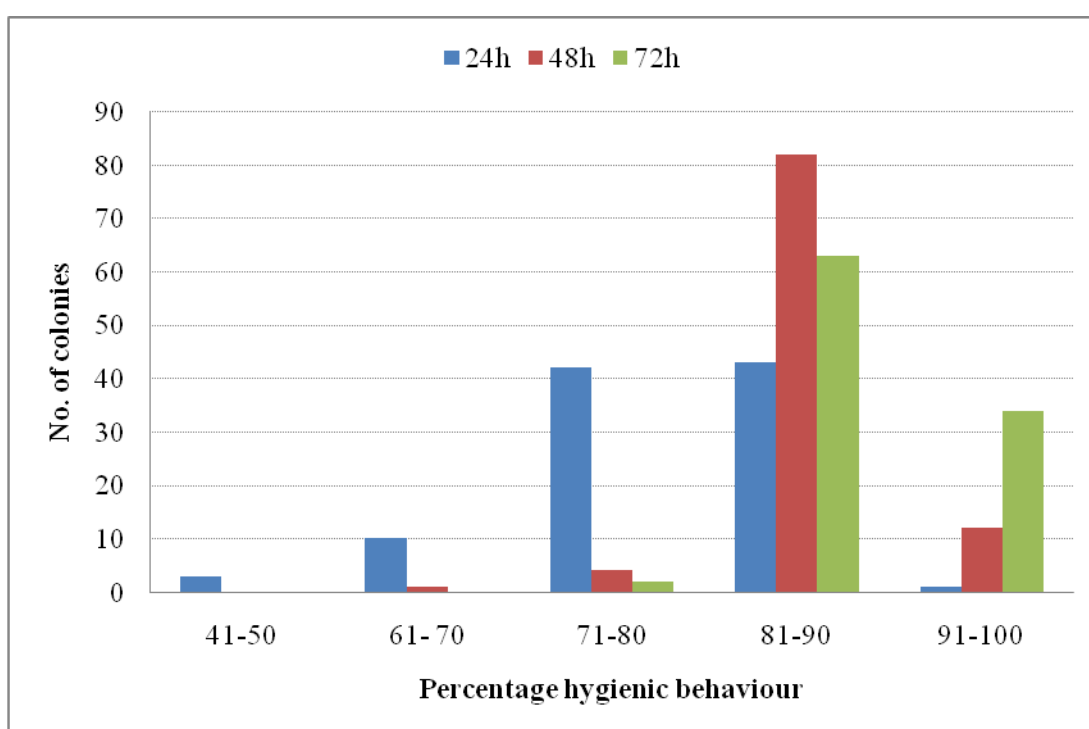


Fig. 1 Number of colonies representing different classes of hygienic behaviour after 24, 48 and 72 h of treatment

Table 14. Percentage of brood cells emptied after 24, 48 and 72h of pricking in three alternate brood cycles

24 h					48 h				72 h			
Col. No.*	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
5 (1H)	83.67 (66.16)	89.33 (70.99)	95.00 (77.42)	89.33 (71.52)	89.33 (70.98)	93.00 (74.65)	96.67 (79.57)	93.00 (75.07)	90.33 (71.92)	93.67 (75.53)	96.67 (79.57)	93.55 (75.67)
15 (2H)	87.67 (69.67)	86.33 (68.32)	91.33 (72.86)	88.44 (70.28)	92.00 (74.35)	90.67 (72.26)	97.67 (81.50)	93.44 (76.03)	94.00 (76.33)	91.67 (73.22)	97.67 (81.50)	94.44 (77.01)
21 (3H)	89.33 (71.16)	90.00 (71.64)	93.67 (75.40)	91.00 (72.74)	93.67 (75.40)	93.67 (75.67)	95.00 (77.16)	94.11 (76.08)	93.67 (75.40)	93.67 (75.67)	95.00 (77.16)	94.11 (76.08)
23 (4H)	87.00 (68.86)	88.00 (69.80)	90.00 (71.59)	88.33 (70.09)	95.67 (78.07)	95.67 (78.07)	90.00 (71.59)	93.78 (75.91)	95.67 (78.07)	95.67 (78.07)	93.33 (75.21)	94.89 (77.12)
25 (5H)	91.33 (72.99)	81.00 (64.15)	87.00 (68.85)	86.44 (68.66)	92.33 (74.19)	93.33 (75.02)	93.33 (75.02)	93.00 (74.74)	94.33 (76.67)	94.67 (76.63)	93.33 (75.02)	94.11 (76.10)
33 (6H)	85.00 (67.19)	91.00 (72.51)	88.00 (69.75)	88.00 (69.82)	91.33 (72.87)	91.00 (72.51)	94.67 (76.63)	92.33 (74.00)	92.33 (73.90)	91.67 (73.22)	94.67 (76.63)	92.89 (74.58)
38 (7H)	82.33 (65.22)	92.67 (74.49)	86.33 (68.33)	87.11 (69.35)	90.33 (71.99)	94.67 (76.67)	91.33 (73.06)	92.11 (73.91)	93.67 (75.43)	94.67 (76.67)	92.67 (74.40)	93.67 (75.50)
31 (1NH)	40.67 (39.59)	44.00 (41.53)	56.00 (48.4)	46.89 (43.18)	64.33 (53.33)	64.00 (53.12)	70.00 (56.79)	66.11 (54.42)	70.67 (57.20)	77.33 (61.57)	75.00 (59.99)	74.33 (59.59)
40 (2NH)	31.33 (34.02)	50.67 (45.36)	60.00 (50.75)	47.33 (43.38)	65.67 (54.31)	73.67 (59.11)	79.00 (62.70)	72.78 (58.71)	73.33 (59.15)	79.67 (63.18)	83.33 (65.88)	78.78 (62.74)
44 (3NH)	39.00 (38.63)	45.67 (42.50)	55.67 (48.23)	46.78 (43.12)	73.00 (58.68)	74.00 (74.37)	72.00 (58.03)	73.00 (58.68)	86.33 (68.31)	81.33 (64.38)	80.00 (63.41)	82.55 (65.37)

*Parentheses are the designations allotted to the selected hygienic and non-hygienic colonies

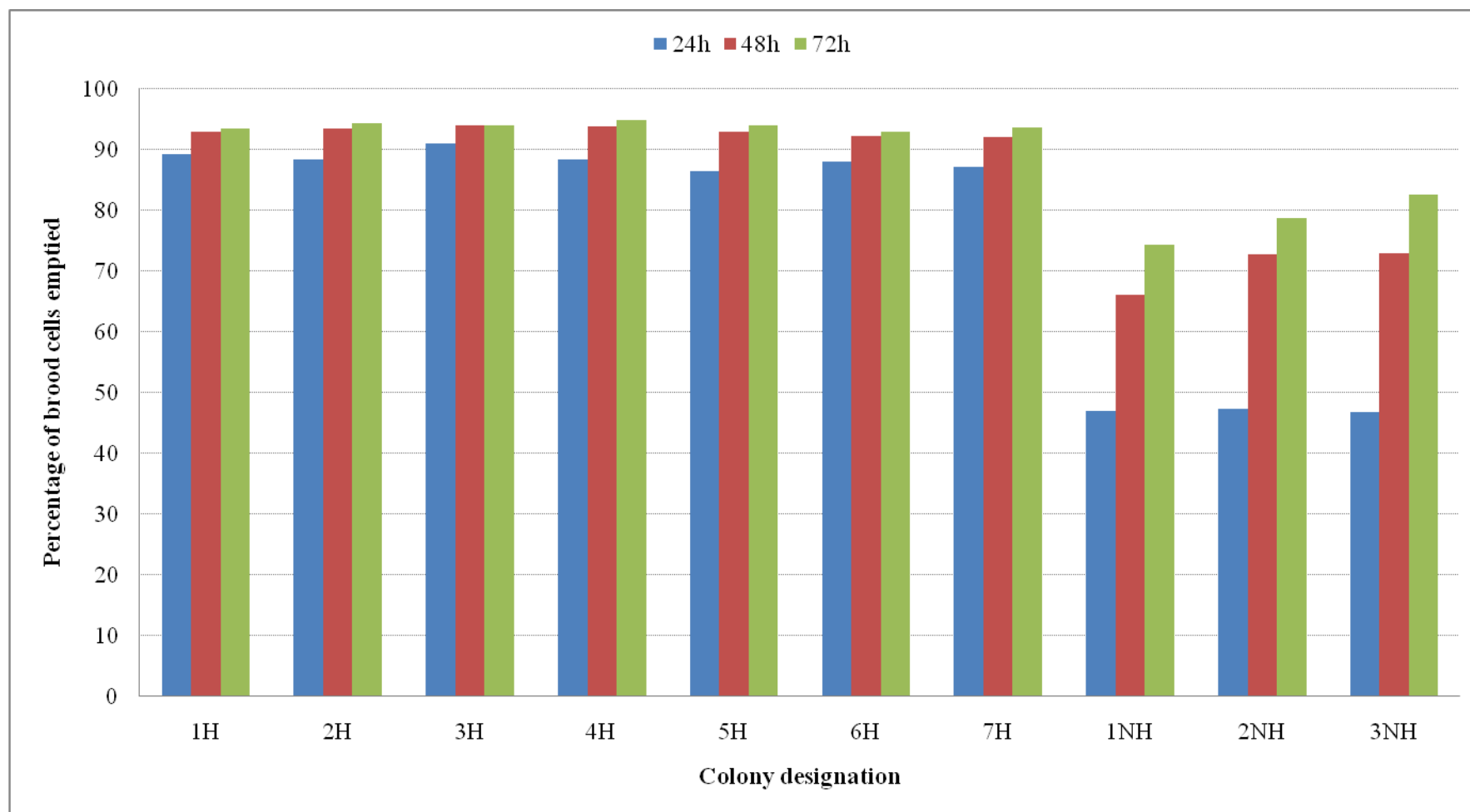


Fig. 2 Mean percentage of brood cells emptied after 24, 48 and 72h of pricking in three alternate brood cycles in the 7 hygienic (1H-7H) and 3 non-hygienic (1NH-3NH) colonies

After 48 h of treatment, the mean percentage of dead brood removal was 92.11- 93.44 per cent in the seven hygienic colonies; all these seven colonies were statistically at par among themselves in this respect. In the three non-hygienic colonies, the mean percentage of dead brood cells emptied had increased to 66.11, 72.78 and 73.00 per cent, respectively, by this time. Among the non-hygienic colonies, colony 1NH was significantly the least hygienic as compared with 2NH and 3NH colonies.

The seven hygienic colonies (1H to 7H) showed the same trend after 72 h, as seen after 48 h of treatment, with no variation among them. On the other hand, all the 3 non-hygienic colonies significantly varied among themselves in removing the dead brood from the pricked brood cells. In contrast to the hygienic colonies (92.89-94.44 per cent), the mean percentage of dead brood removed ranged from 74.33-82.55 per cent in the non-hygienic colonies.

The mean percentage of pricked brood cells uncapped (Table 15, Fig. 3), ranged from 1.44-4.67 per cent after 24 h of treatment in the seven hygienic colonies as compared with the three non-hygienic colonies where only uncapping of pricked brood cells was recorded to range between from 9.56-20.89 per cent. Colony 2H was at par with colony 7H., while colonies 3H, 6H and 4H formed a separate group and colonies 1H and 5H formed another statistically distant group. Colonies 1NH and 2NH were at par with each other whereas colony 3NH varied significantly from the above two colonies.

After 48 h of treatment the maximum of dead brood had found to have been removed by the seven hygienic colonies and the percentage of dead brood cells only uncapped were in the range of 0.22-2.00 per cent, and in the three non-hygienic colonies, it ranged from 2.89-10.44 per cent. Colonies 3H, 4H, 6H and 7H were at par with each other and formed one group the colonies 1H, 2H and 5H formed another group. 3NH and 2 NH were at par and varied significantly from 1NH and the latter recording lesser uncapping. Similarly 72 h after treatment in the hygienic colonies, the maximum of 1.56 per cent brood cells only were uncapped and colonies 3H, 4H, 6H and 7H were at par among themselves in this respect. In non-hygienic colonies, 3.44 -4.78 per cent uncapping of dead brood cells was observed and all the three colonies were at par in this respect.

In all the hygienic colonies, after 24 h of the treatment, dead brood cells remaining capped were significantly less than in the non-hygienic colonies. A minimum of 6.00 per cent (colony 3H) and a maximum of 12.78 mean per cent (colony 5H) of dead brood cells remained capped among the seven hygienic colonies, while the non-hygienic colonies significantly varied among themselves and the brood cells remaining capped ranged between 32.33-43.56 per cent. In colony 3H significantly lesser brood cells remained capped and colonies 1H, 2H, 4H, 6H and 7H formed another statistical group.

Table 15. Percentage of brood cells only uncapped after 24, 48 and 72h of pricking in three alternate brood cycles

24h					48h				72h			
Col. No. *	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
5 (1H)	0.33 (1.91)	2.67 (7.63)	1.33 (5.42)	1.44 (4.99)	0.00 (0.00)	0.67 (2.71)	0.00 (0.00)	0.22 (0.90)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
15 (2H)	4.67 (12.41)	5.00 (12.87)	2.00 (7.94)	3.89 (11.08)	1.33 (2.71)	0.67 (2.71)	0.00 (0.00)	0.67 (2.18)	0.00 (0.00)	0.33 (1.91)	0.00 (0.00)	0.11 (0.64)
21 (3H)	2.33 (7.01)	4.67 (12.28)	2.00 (6.55)	3.00 (8.62)	0.00 (0.00)	3.33 (10.40)	1.33 (5.42)	1.56 (5.27)	0.00 (0.00)	3.33 (10.40)	1.33 (5.42)	1.56 (5.27)
23 (4H)	4.00 (11.37)	3.00 (8.05)	0.67 (3.82)	2.56 (7.75)	0.67 (3.82)	0.67 (3.82)	0.67 (3.82)	0.67 (3.82)	0.67 (3.82)	0.67 (3.82)	0.67 (2.71)	0.67 (3.45)
25 (5H)	3.67 (11.01)	1.33 (3.84)	0.00 (0.00)	1.67 (4.95)	0.67 (2.71)	0.00 (0.00)	0.00 (0.00)	0.22 (0.90)	0.33 (1.91)	0.00 (0.00)	0.00 (0.00)	0.11 (0.64)
33 (6H)	3.00 (9.72)	0.00 (0.00)	6.67 (14.92)	3.22 (8.22)	1.00 (3.32)	0.67 (2.71)	1.33 (6.53)	1.00 (4.19)	0.67 (2.71)	0.67 (2.71)	1.33 (6.53)	0.89 (3.98)
38 (7H)	6.67 (14.95)	0.67 (2.71)	6.67 (14.85)	4.67 (10.83)	2.67 (9.36)	0.00 (0.00)	3.33 (10.06)	2.00 (6.47)	1.67 (6.03)	0.00 (0.00)	2.00 (6.22)	1.22 (4.08)
31 (1NH)	9.33 (17.70)	8.67 (17.09)	10.67 (18.98)	9.56 (17.92)	4.67 (11.56)	2.00 (8.13)	2.00 (8.13)	2.89 (8.63)	3.33 (6.14)	0.00 (0.00)	5.67 (13.68)	4.78 (9.23)
40 (2NH)	9.33 (17.70)	7.33 (15.65)	22.67 (28.40)	13.11 (20.58)	9.67 (17.05)	9.33 (17.30)	6.67 (14.95)	8.55 (16.43)	5.67 (11.29)	0.00 (0.00)	6.00 (14.07)	3.89 (8.45)
44 (3NH)	5.00 (12.80)	37.33 (37.64)	20.33 (26.78)	20.89 (25.74)	13.00 (21.13)	12.33 (20.55)	6.00 (14.04)	10.44 (18.57)	2.00 (6.65)	5.00 (12.92)	3.33 (10.34)	3.44 (9.97)

*Parentheses are the designations allotted to the selected hygienic and non-hygienic colonies

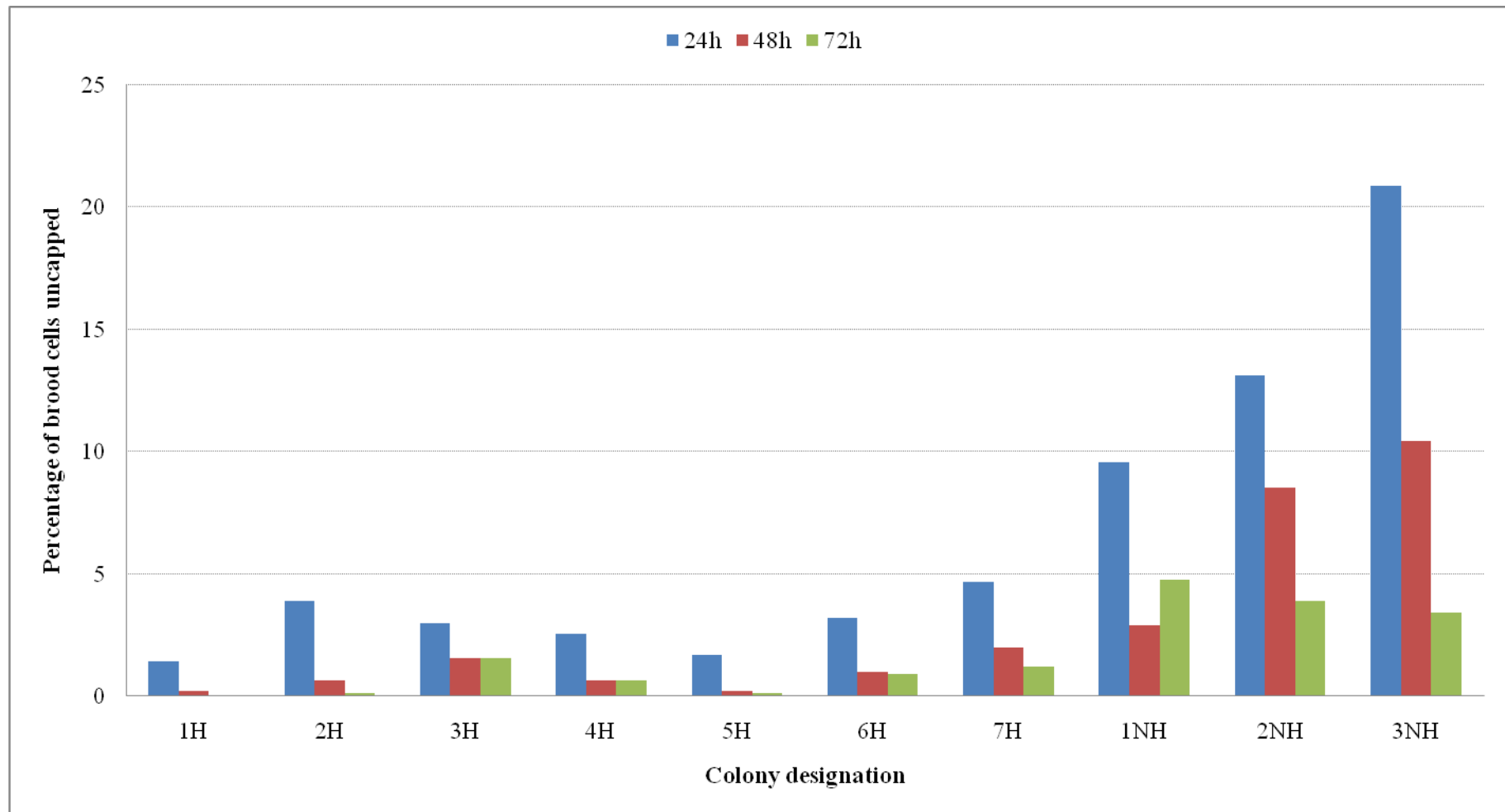


Fig. 3 Mean percentage of brood cells only uncapped (dead brood not removed) after 24, 48 and 72 h of pricking over the three alternate brood cycles in the seven hygienic (1H-7H) and three non-hygienic (1NH-3NH) colonies

After 48 hours of treatment, the cells remaining capped ranged between 4.33 (colony 3H) to 6.78 per cent (colony 5H). Colonies 2H, 3H and 4H were at par among themselves and the remaining four hygienic colonies were at par among themselves. Colonies 2NH and 3NH were statistically at par with each other recording mean percentage of capped brood cells being 19.78 and 16.56 per cent, respectively and significantly varied from colony 1NH where 31.00 per cent of brood cells remained capped.

In hygienic colonies, 4-33 to 6.44 per cent of brood cells remained capped after 72 h of pricking and colonies 2H, 3H, 4H, 5H and 7H were at par among themselves. In non- hygienic colonies, similar to 48 h of treatment, 2NH and 3NH were at par and in colony 1NH capped brood cells percentage decreased to 23 per cent (Table 16, Fig. 4).

The present findings are in line with the findings of the studies conducted by different scientists (Spivak and Gilliam 1993, Kefuss *et al* 1996, Spivak and Downey 1998, Arathi and Spivak 2001, Panasiuk *et al* 2008, Bāk *et al* 2010) who reported that hygienic bees removed higher proportion of dead brood compared to non-hygienic within the same time frame. Guerra (2000) reported that Africanized colonies removed a mean of 51per cent of the infested brood, while Italian colonies removed 25per cent. Similarly, when Africanized colonies were compared with colonies of Italian bees from Fernando de Noronha, the former were found to be significantly more efficient at removing infested brood (61 vs. 35%, respectively). Balhareth *et al* (2012) reported that after 24 h, the general mean of dead brood removal in *A.mellifera jemenitica* was significantly higher (85.28%) than that of *A. m. carnica* (43.84%). After 48 h, workers of *A.m. jemenitica* removed 97.60 per cent of brood, while those of *A. m. carnica* removed 79.32 per cent with significant difference between the two. After 72 h, the brood removal rates were 99.78 and 93.29 per cent for *A.m. jemenitica* and *A. m. carnica*, respectively. As in the present studies, it was noted that time needed to detect and remove brood in hygienic and non-hygienic colonies was variable. Hygienic behaviour was evaluated in 45 production colonies under the Australian Honey Bee Breeding Program, Tasmania, Australia and it was found that 20 per cent of the total colonies tested were 90-100 per cent hygienic, averaging 96 per cent hygienic, 33 per cent colonies tested between 70-89 per cent, averaging 78 per cent hygienic; and 47 per cent tested below 70 per cent averaging 47 per cent hygienic (Anonymous 2015).

Table 16. Percentage of brood cells remaining capped after 24, 48 and 72h of pricking in three alternate brood cycles

24h					48h				72h			
Col. No. *	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
5 (1H)	16.00 (23.52)	8.00 (16.42)	3.67 (10.86)	9.22 (16.93)	10.67 (18.98)	6.33 (14.43)	3.33 (10.40)	6.78 (14.60)	9.67 (18.05)	6.33 (14.43)	3.33 (10.40)	6.44 (14.29)
15 (2H)	7.67 (15.71)	8.67 (17.07)	6.67 (14.89)	7.67 (15.89)	6.67 (14.27)	7.67 (16.06)	2.33 (8.47)	5.56 (12.93)	6.00 (13.64)	7.67 (16.06)	2.33 (8.47)	5.33 (12.72)
21 (3H)	8.33 (16.68)	5.33 (13.16)	4.33 (11.66)	6.00 (13.84)	6.33 (14.56)	3.00 (8.05)	3.67 (10.53)	4.33 (11.05)	6.33 (14.56)	3.00 (8.05)	3.67 (10.53)	4.33 (11.05)
23 (4H)	9.00 (17.45)	9.00 (17.45)	9.33 (17.75)	9.11 (17.55)	3.67 (10.95)	3.67 (10.95)	9.33 (17.75)	5.56 (13.22)	3.67 (10.95)	3.67 (10.95)	6.00 (14.08)	4.44 (12.00)
25 (5H)	22.00 (27.95)	7.33 (15.65)	9.00 (17.29)	12.78 (20.30)	3.67 (10.95)	3.67 (10.95)	9.33 (17.75)	5.56 (13.22)	5.33 (12.50)	5.33 (13.33)	6.67 (14.95)	5.78 (13.59)
33 (6H)	12.00 (20.23)	9.00 (17.45)	4.00 (11.53)	8.33 (16.40)	7.67 (16.06)	8.33 (16.75)	4.00 (11.53)	6.67 (14.78)	7.00 (15.25)	7.67 (16.04)	4.00 (11.53)	6.22 (14.28)
38 (7H)	11.00 (19.20)	6.67 (14.77)	7.00 (15.31)	8.22 (16.43)	7.00 (15.17)	5.33 (13.29)	5.33 (13.26)	5.89 (13.91)	4.67 (12.28)	5.33 (13.29)	5.33 (13.26)	5.11 (12.94)
31 (1NH)	50.00 (44.98)	47.33 (43.45)	33.33 (35.25)	43.56 (41.22)	31.00 (33.68)	34.00 (35.64)	28.00 (31.93)	31.00 (33.75)	26.00 (30.40)	22.67 (28.39)	20.33 (26.79)	23.00 (28.53)
40 (2NH)	59.33 (50.37)	42.00 (40.38)	17.33 (24.56)	39.56 (38.44)	24.67 (29.70)	20.33 (26.79)	14.33 (22.24)	19.78 (26.24)	21.00 (27.18)	20.33 (26.79)	10.67 (19.04)	17.33 (24.33)
44 (3NH)	56.00 (48.43)	17.00 (11.89)	24.00 (29.31)	32.33 (34.02)	14.00 (26.66)	13.67 (9.88)	22.00 (27.96)	16.56 (23.86)	11.67 (15.19)	13.67 (11.28)	16.67 (17.43)	14.00 (21.90)

*Parentheses are the designations allotted to the selected hygienic and non-hygienic colonies

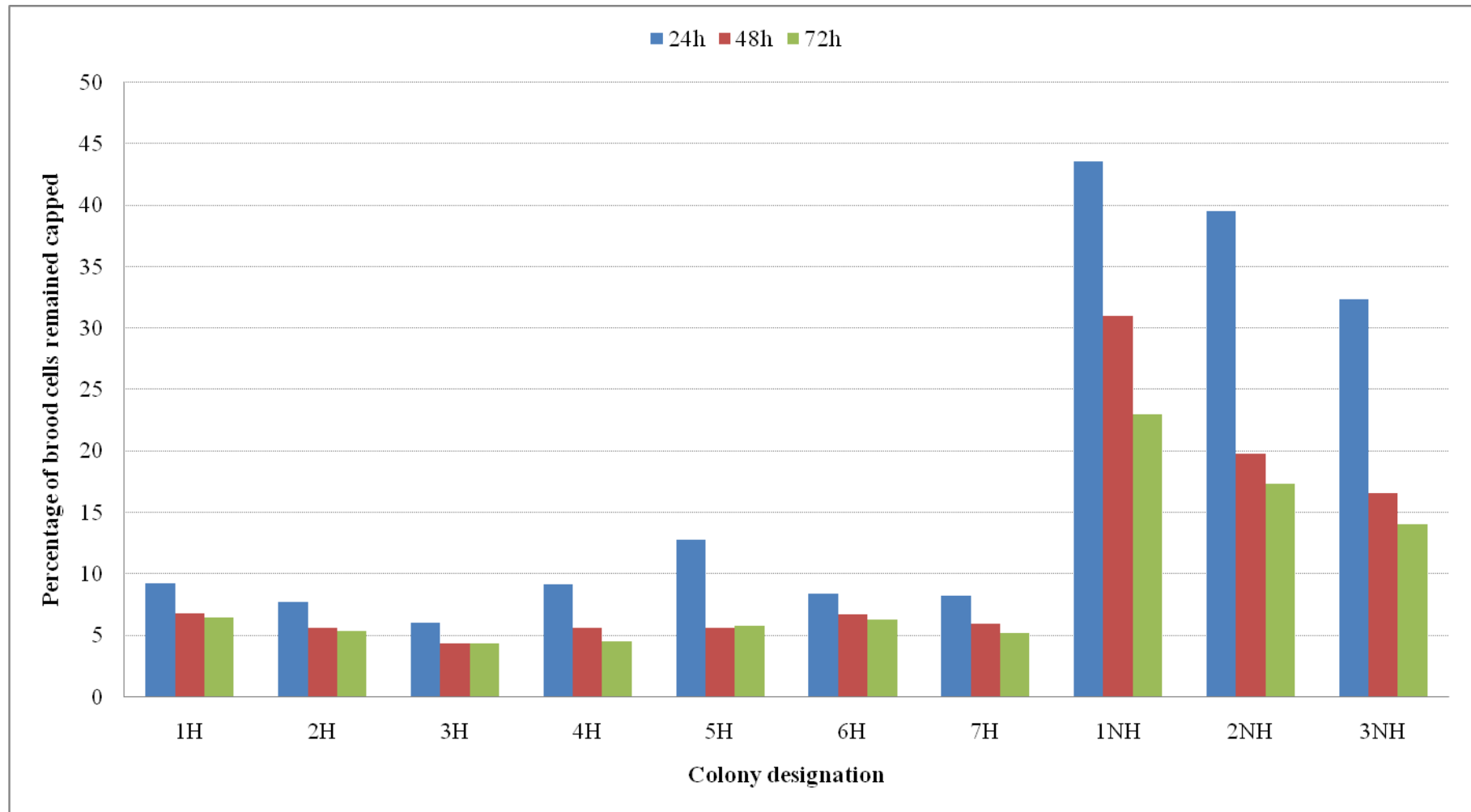


Fig. 4 Mean percentage of brood cells remaining capped after 24, 48 and 72 h of pricking over the three alternate brood cycles in the 7 hygienic (1H-7H) and three non-hygienic (1NH-3NH) colonies

4.2 Evaluation of the selected 10 honey bee colonies (7 hygienic and 3 non- hygienic) for hygienic behaviour

For establishing the righteous selection of the colonies through confirming the consistency of hygienic response among the selected ten colonies, intensive hygienic response data were collected for three days over three brood cycles. The methodology followed for the Experiment 4.1, was adopted

4.2.1 Dead brood emptied after 24, 48 and 72 h of pricking in three alternate brood cycles

The pin killed brood cells emptied (Table 17, Fig. 5) within 24 h of pricking, in the first brood cycle, ranged from 42.67-93.67 per cent and in the second brood cycle it was 44.0-93.67 and 41.33-93.67 per cent in the third alternate brood cycle. The overall mean dead brood removal was 88.93 per cent (range: 85.11-93.33 %) in the seven hygienic colonies and 50.11 per cent (range: 42.67-56.67 %) in non-hygienic colonies. Among the hygienic colonies, colonies 2H, 3H and 4H were at par with each other and statistically recorded higher dead brood removal than the remaining four colonies. The three non-hygienic colonies too showed variability among the colonies.

After 48 h, in the first test brood cycle, the dead brood cells emptied ranged from 86.67-93.67 per cent. In the second and third alternate test brood cycles, the brood cells emptied were in the range of 91.00-97.67 and 88.67-96.33 per cent, respectively. The mean pricked brood removal was 93.28 per cent (range: 91.78-95.11%) in the seven hygienic colonies and 72.89 per cent (range: 63.56-78.33%) in the non-hygienic colonies. Colonies 2H and 4H were at par with each other and showed significantly high hygienic behaviour than 3H, 6H and 7H colonies. Among the three non-hygienic colonies, 2NH and 3NH were at par with each other and colony 1NH was the least hygienic.

After 72 h, the dead brood removal ranged from 89.33-94.67 per cent in the seven hygienic colonies and 72.33 -90.00 per cent in non-hygienic colonies in the first test brood cycle. It was 92.00-97.67 and 91.67-96.67 per cent in the second and third test brood cycle, respectively. The hygienic behaviour was an average of 94.21 per cent (range: 93.00-95.67 %) and 81.33 per cent (range: 75.89-84.33 %) per cent in the seven hygienic and three non-hygienic colonies, respectively.

4.2.2 Dead brood cells only uncapped after 24, 48 and 72 h of pricking in three alternate brood cycles

The pin killed brood cells only uncapped (Table 18, Fig. 6), in the first test brood cycle were minimal (9.00%) among the hygienic colonies and were in the range of 15.00-19.33 per cent among the non-hygienic colonies after 24 h of pricking. In the second and third test brood cycle it was 0.67-12.33 and 23.33-30.33 per cent, and 3.00-14.67 and 20.33-31.67 per cent, respectively among hygienic and non-hygienic colonies.

Table 17. Percentage of brood cells emptied after 24, 48 and 72h of pricking in three alternate brood cycles*

Col. No.	24h				48h				72h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	90.00±0.58 (71.55)	81.00±0.58 (64.14)	84.33±1.67 (66.71)	85.11±2.63 (67.47)	93.00±1.15 (74.73)	93.67±0.67 (75.43)	88.67±0.88 (70.33)	91.78±1.57 (73.50)	93.67±0.67 (75.43)	93.67±0.67 (75.43)	91.67±0.88 (73.23)	93.00±0.67 (74.70)
2H	93.67±0.88 (75.46)	92.67±1.20 (74.37)	93.67±1.86 (75.67)	93.33±0.33 (75.17)	93.67±0.88 (75.46)	94.00±1.53 (76.05)	96.00±0.58 (78.49)	94.56±0.73 (76.67)	93.67±0.88 (75.46)	94.00±1.53 (76.05)	96.00±0.58 (78.49)	94.56± 0.73 (76.67)
3H	92.67±0.67 (74.29)	90.67±0.67 (72.20)	93.33±0.33 (75.02)	92.22±0.80 (73.84)	92.67±0.67 (74.29)	93.00±1.00 (74.71)	95.67±0.67 (78.03)	93.78±0.95 (75.68)	93.67±0.67 (75.43)	93.00±1.00 (74.71)	95.67±0.67 (78.03)	94.11±0.80 (76.06)
4H	90.67±0.67 (72.20)	93.67±2.73 (76.43)	93.33±0.33 (75.02)	92.56±0.95 (74.55)	91.33±1.33 (72.95)	97.67±0.88 (81.50)	96.33±0.33 (78.95)	95.11±1.93 (77.80)	93.00±0.58 (74.65)	97.67±0.88 (81.50)	96.33±0.33 (78.95)	95.67±1.39 (78.37)
5H	90.33±1.45 (71.96)	86.00±1.15 (68.03)	80.00±1.00 (63.042)	85.44±3.00 (67.80)	92.67±0.88 (74.31)	91.00±1.53 (72.62)	91.67±1.86 (73.37)	91.78±0.98 (73.44)	92.67±0.88 (74.31)	92.00±0.58 (73.56)	94.67±1.33 (76.80)	93.11±0.80 (74.89)
6.H	84.33±0.88 (66.67)	90.00±0.00 (71.54)	90.00±1.73 (71.67)	88.11±1.85 (69.96)	86.67±0.88 (68.58)	95.00±1.15 (77.22)	95.67±1.86 (78.46)	92.44±2.90 (74.75)	89.33±0.67 (70.93)	95.00±1.15 (77.22)	96.67±0.88 (79.63)	93.67 ±2.22 (75.92)
7H	85.00±0.58 (67.19)	81.00±0.58 (64.14)	91.33±1.45 (72.97)	85.78±3.01 (68.10)	93.00±1.53 (74.79)	92.33±0.33 (73.90)	95.33±1.67 (77.84)	93.56±0.91 (75.51)	94.67±0.33 (76.63)	95.00±0.00 (77.05)	96.33±0.67 (79.01)	95.33± 0.51 (77.56)
1NH	42.67±1.45 (40.76)	44.00±1.15 (41.54)	41.33±0.33 (39.99)	42.67±0.77 (40.76)	64.00±1.15 (53.11)	64.00±1.15 (53.11)	62.67±0.33 (52.32)	63.56±0.44 (52.85)	72.33±0.88 (58.25)	80.00±1.73 (63.45)	75.33±0.33 (60.20)	75.89± 2.23 (60.63)
2NH	54.00±0.58 (47.28)	47.00±1.73 (43.26)	52.00±1.15 (46.13)	51.00±2.08 (45.55)	81.67±1.86 (64.67)	71.00±0.58 (57.40)	77.67±1.20 (61.79)	76.78±3.11 (61.29)	90.00±0.00 (71.54)	80.00±0.00 (63.41)	83.00±0.58 (65.63)	84.33± 2.96 (66.86)
3NH	55.00±0.58 (47.85)	51.33±0.33 (45.75)	63.67±1.20 (52.92)	56.67±3.66 (48.84)	78.00±1.00 (62.01)	75.67±2.03 (60.46)	81.33±0.67 (64.38)	78.33±1.64 (62.28)	80.33±0.88 (63.66)	87.00±1.15 (68.88)	84.00±0.58 (66.40)	83.78± 1.93 (66.31)
Mean	77.83 (63.52)	75.73 (62.14)	78.30 (63.95)	-	86.67 (69.49)	86.73 (70.24)	88.10 (71.40)	-	89.33 (71.63)	90.73 (73.13)	90.97 (73.64)	-
LSD (p=0.05)	Colony (1.88) Brood Cycle (1.03) Colony x Brood Cycle (3.25)				Colony (2.03) Brood Cycle (1.11) Colony x Brood Cycle (3.52)				Colony (1.57) Brood Cycle (0.86) Colony x Brood Cycle (2.73)			

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

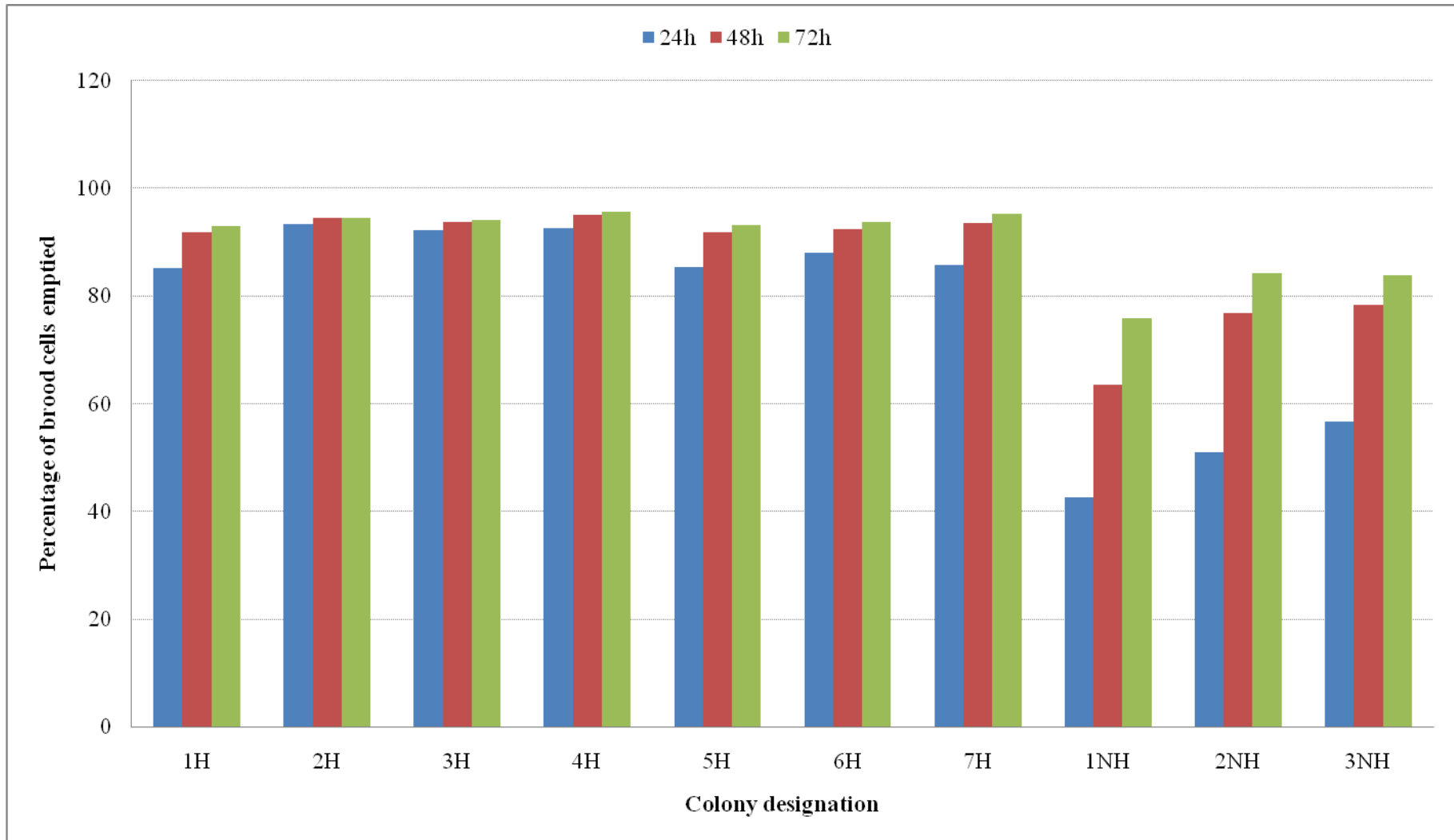


Fig. 5 Mean percentage of brood cells emptied after 24, 48 and 72 h of pricking in three alternate brood cycles in the seven hygienic (1H-7H) and three non-hygienic (1NH-3NH) colonies

Table 18. Percentage of brood cells only uncapped after 24, 48 and 72h of pricking in three alternate brood cycles*

Col. No.	24 h				48 h				72 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	3.67±0.88 (10.86)	7.33±1.76 (15.46)	4.67±1.76 (12.03)	5.22 (12.78)	0.67±0.67 (2.71)	0.67±0.67 (2.71)	2.00±1.15 (6.55)	1.11 (3.99)	0.00±0.00 (2.71)	0.67±0.67 (2.71)	0.67±0.67 (6.55)	0.44 (3.99)
2H	0.00±0.00 (0.00)	0.67±0.67 (2.71)	3.33±0.67 (10.40)	1.33 (4.37)	0.00±0.0 (0.00)	0.00±0.00 (0.00)	1.33±1.33 (3.84)	0.44 (1.28)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	1.33±1.33 (3.84)	0.44 (1.28)
3H	0.00±0.00 (0.00)	0.67±0.67 (2.71)	3.67±0.33 (11.01)	1.44 (4.57)	1.33±0.33 (6.53)	0.00±0.00 (0.00)	1.33±0.67 (5.42)	0.89 (3.98)	0.33±0.33 (6.53)	0.00±0.00 (0.00)	1.33±0.67 (5.42)	0.56 (3.98)
4H	0.67±0.67 (2.71)	1.67±0.88 (6.03)	3.67±0.33 (11.01)	2.00 (6.58)	1.67±0.88 (6.03)	0.00±0.00 (0.00)	2.33±0.67 (8.56)	1.33 (4.86)	0.00±0.00 (6.03)	0.00±0.00 (0.00)	2.33±0.67 (8.56)	0.78 (4.86)
5H	0.00±0.00 (0.00)	4.33±0.67 (11.93)	14.67±1.20 (22.48)	6.33 (11.47)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	3.67±0.33 (11.01)	1.22 (3.67)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.67±0.33 (11.01)	0.22 (3.67)
6.H	0.00±0.00 (0.00)	1.33±0.33 (6.53)	5.33±0.88 (13.26)	2.22 (6.60)	2.67±0.33 (9.36)	0.67±0.67 (2.71)	2.33±1.20 (7.17)	1.89 (6.41)	0.00±0.00 (9.36)	0.67±0.67 (2.71)	2.00±0.58 (7.17)	0.89 (6.41)
7H	9.00±0.58 (17.43)	12.33±0.33 (20.55)	3.00±0.00 (9.97)	8.11 (15.98)	1.67±1.67 (4.31)	2.67±0.33 (9.36)	2.00±1.00 (6.65)	2.11 (6.77)	0.00±0.00 (4.31)	0.00±0.00 (9.36)	1.00±1.00 (6.65)	0.33 (6.77)
1NH	15.00±2.65 (22.61)	30.33±1.76 (33.39)	31.67±0.67 (34.23)	25.67 (30.08)	6.00±0.58 (14.14)	18.00±0.58 (25.09)	14.67±0.67 (22.50)	12.89 (20.58)	1.00±0.00 (14.14)	2.00±1.15 (25.09)	5.67±0.67 (22.50)	2.89 (20.58)
2NH	19.33±1.20 (26.05)	25.67±0.33 (30.43)	25.33±1.33 (30.19)	23.44 (28.89)	1.33±1.33 (3.84)	6.00±0.58 (14.14)	4.00±1.00 (11.37)	3.78 (9.79)	0.00±0.00 (3.84)	2.00±1.15 (14.14)	1.67±0.67 (11.37)	1.22 (9.79)
3NH	19.00±1.00 (25.82)	23.33±0.33 (28.87)	20.33±0.33 (26.79)	20.89 (27.16)	2.67±0.33 (9.36)	2.33±0.88 (8.47)	5.67±0.33 (13.75)	3.56 (10.53)	1.00±1.00 (9.36)	0.67±0.67 (8.47)	3.00±0.00 (13.75)	1.56 (10.53)
Mean	6.67 (10.55)	10.77 (15.86)	11.57 (18.14)	-	1.80 (5.63)	3.03 (6.25)	3.93 (9.68)	-	0.23 (5.63)	0.60 (6.25)	1.97 (9.68)	-
LSD (p=0.05)	Colony (2.27) Brood Cycle (1.24) Colony x Brood Cycle (3.93)				Colony (3.44) Brood Cycle (1.88) Colony x Brood Cycle (5.96)				Colony (3.13) Brood Cycle (1.72) Colony x Brood Cycle NS			

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

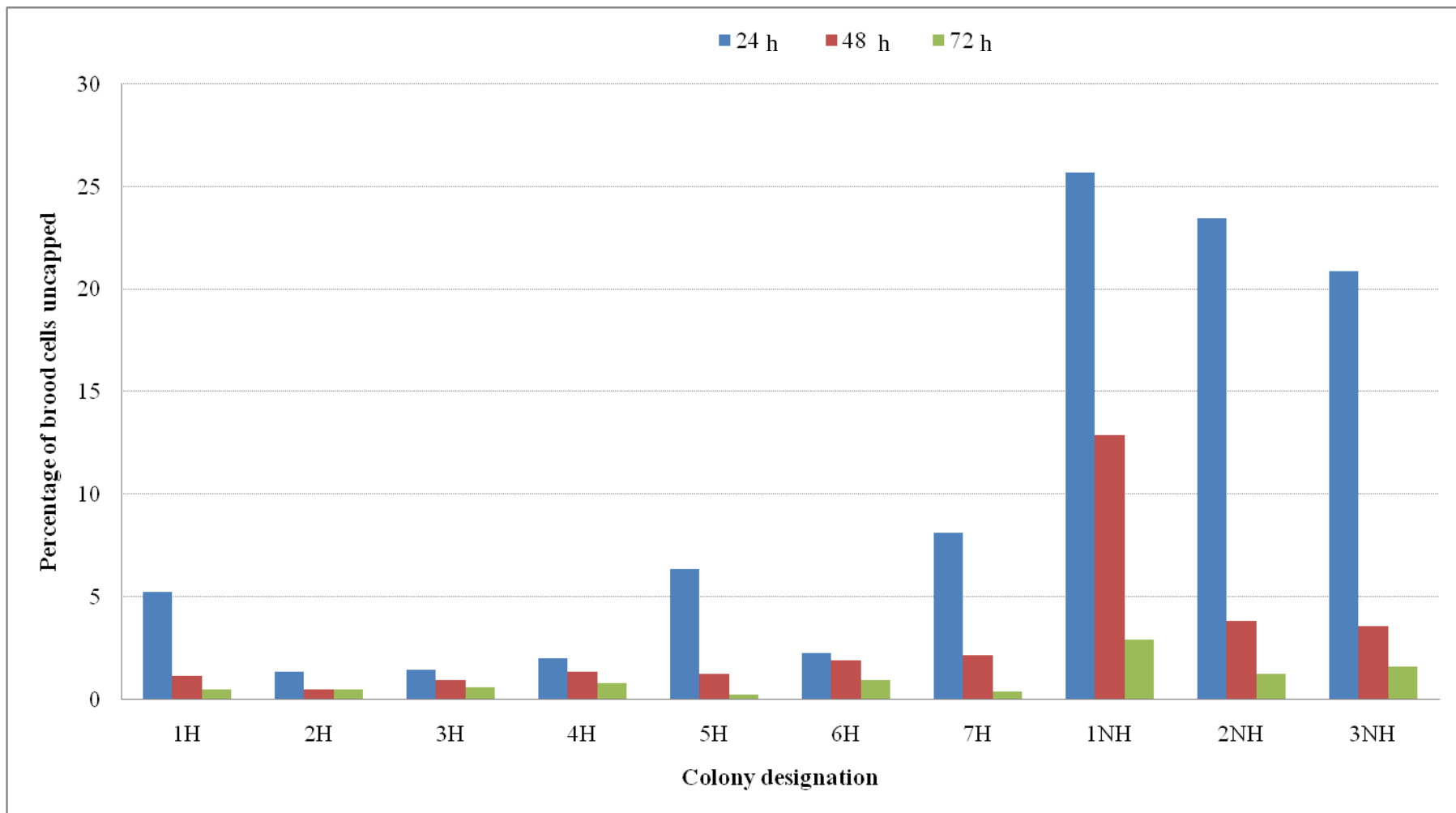


Fig. 6 Mean percentage of brood cells uncapped after 24, 48 and 72 h of pricking in three alternate brood cycles in the seven hygienic (1H-7H) and three non-hygienic (1NH-3NH) colonies

The overall mean of dead brood cells, that were only uncapped, was 2.85 per cent (range: 1.33 to 8.11%) among the seven hygienic colonies and 23.33 per cent (range: 20.89- 25.67%) among the three non-hygienic colonies. Pricked brood cells only uncapped were low in the hygienic colonies, thus this character will help in preventing the spread of diseases. Among the three non-hygienic colonies uncapping of dead brood sans removal was significantly higher as compared with those among the hygienic colonies.

The uncapped cells were further cleaned and thus the percentage of only uncapped brood cells decreased in all the 10 colonies 48 h after treatment. In the hygienic colonies only uncapped brood was in the range of 0-2.67, 0-2.67 and 1.33-2.33 per cent, for the I, II and III test brood cycles, and 1.33-6.00, 2.33-18.00 and 4.00-14.67 per cent, respectively among the non-hygienic colonies. In the hygienic colonies, overall mean of dead brood cells only uncapped was 1.28 per cent (range: 0.44-2.11%) and 6.74 per cent (range: 3.56 to 12.89 %) in the non-hygienic colonies. Colonies 1H-5H recorded least percentage of uncapped brood cells and were statistically at par with each other. After 72 h, only uncapping of dead brood cells ranged between 0.22-0.89 per cent in the hygienic colonies and 1.22-2.89 in the non-hygienic colonies

4.2.3 Dead brood cells remaining capped after 24, 48 and 72 h of pricking in three alternate brood cycles

A perusal of data recorded in Table 19 and Fig. 7, reveals that after 24 h of pricking, in the hygienic colonies, the pricked brood cells remaining capped, in all the three individual alternate brood cycles, were in the range of 6.00-15.67, 4.67-11.67 and 3.00-11.00 per cent, respectively. On the other hand, in non-hygienic colonies, it ranged between 26.00-42.33, 25.33-27.33 and 16.00-27.67 per cent, respectively. The mean dead brood cells remaining capped in hygienic colonies was 7.25 per cent (range: 5.33-9.67 %) and in non-hygienic colonies mean brood cells remained capped were 26.63 per cent (range: 22.44-31.89 %).

At 48 h of pricking, dead brood cells remaining capped though had decreased after 24 h in all the 10 colonies, yet the non-hygienic colonies recorded significantly higher percentage of capped cells as compared to the hygienic ones both in the individual brood cycles and in the overall mean of all the three brood cycles.

After 72 h, in the hygienic colonies for all the three test brood cycles and in overall mean there was not much difference in the mean per cent of dead brood cells remaining capped. In non-hygienic colonies among the three individual test brood cycles, the brood cells remaining capped ranged between 10.00-26.67, 12.33-18.00 and 13.00-19.00 per cent. The overall mean of brood cells remaining capped was 5.27 per cent (range: 3.56-6.67%) in hygienic colonies, and 16.78 per cent (range: 14.44-21.22 %) in non- hygienic colonies.

Thus the seven hygienic and three non-hygienic colonies selected from field studies showed consistency in their hygienic traits and these colonies were therefore, used for collecting the samples of adult honey bee workers for genotyping with the SSR markers reported to be linked with hygienic behaviour and the genetic diversity studies were also carried on these selected colonies.

Table 19. Percentage of brood cells remaining capped after 24, 48 and 72h of pricking in three alternate brood cycles*

Col. No.	24 h				48 h				72 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	6.33±0.67 (14.53)	11.67±1.67 (19.88)	11.00±0.58 (19.35)	9.67 (17.92) ^c	6.33±0.67 (14.53)	5.67±0.67 (13.72)	9.33±0.33 (17.78)	7.11 (14.13)	6.33±0.67 (14.53)	5.67±0.67 (13.72)	7.67±0.33 (16.06)	6.56 (14.77)
2H	6.33±0.88 (14.50)	6.67±0.88 (14.89)	3.00±1.73 (8.05)	5.33 (12.48)	6.33±0.88 (14.50)	6.00±1.53 (13.91)	2.67±1.45 (7.63)	5.00 (14.21)	6.33±0.88 (14.50)	6.00±1.53 (13.91)	2.67±1.45 (7.63)	5.00 (12.01)
3H	7.33±0.67 (15.678)	8.67±0.67 (17.09)	3.00±0.00 (9.97)	6.33 (14.24)	6.00±0.58 (14.14)	7.00±1.00 (15.25)	3.00±0.00 (9.97)	5.33 (14.70)	6.00±0.58 (14.14)	7.00±1.00 (15.25)	3.00±0.00 (9.97)	5.33 (13.12)
4H	8.67±1.33 (17.01)	4.67±1.86 (11.75)	3.00±0.00 (9.97)	5.44 (12.91)	7.00±0.58 (15.31)	2.33±0.88 (8.47)	1.33±0.88 (5.24)	3.56 (11.89)	7.00±0.58 (15.31)	2.33±0.88 (8.47)	1.33±0.88 (5.24)	3.56 (9.67)
5H	9.67±1.45 (18.01)	9.67±1.76 (17.97)	5.33±1.33 (13.16)	8.22 (16.38)	7.33±0.88 (15.65)	9.00±1.53 (17.34)	4.67±1.67 (12.12)	7.00 (16.50)	7.33±0.88 (15.65)	8.00±0.58 (16.40)	4.67±1.67 (12.12)	6.67 (14.73)
6.H	15.67±0.88 (23.29)	8.67±0.33 (17.11)	4.67±1.33 (12.16)	9.67 (17.52)	10.67±0.67 (19.04)	4.33±0.67 (11.93)	2.00±1.15 (6.55)	5.67 (15.49)	10.67±0.67 (19.04)	4.33±0.67 (11.93)	1.33±1.33 (3.84)	5.44 (11.61)
7H	6.00±0.58 (14.14)	6.67±0.33 (14.95)	5.67±1.45 (13.52)	6.11 (14.20)	5.33±0.33 (13.33)	5.00±0.00 (12.92)	2.67±0.00 (7.63)	4.33 (13.13)	5.33±0.33 (13.33)	5.00±0.00 (12.92)	2.67±1.45 (7.63)	4.33 (11.29)
1NH	42.33±2.19 (40.56)	25.67±0.67 (30.42)	27.67±1.45 (31.71)	31.89 (34.23)	30.00±1.45 (33.19)	18.00±1.00 (25.09)	22.67±0.58 (28.41)	23.56 (29.14)	26.67±0.88 (31.07)	18.00±0.58 (25.09)	19.00±1.00 (25.82)	21.22 (27.33)
2NH	26.67±1.2 (31.07)	27.33±1.45 (31.49)	22.67±0.67 (28.41)	25.56 (30.33)	13.67±0.88 (21.65)	23.00±1.20 (28.64)	18.33±1.00 (25.33)	18.33 (25.14)	10.00±0.00 (18.43)	18.00±1.15 (25.07)	15.33±0.33 (23.04)	14.44 (22.18)
3NH	26.00±1.53 (30.63)	25.33±0.33 (30.21)	16.00±1.53 (23.52)	22.44 (28.12)	19.33±0.88 (26.05)	18.67±1.20 (25.58)	13.00±0.88 (21.12)	17.00 (25.81)	18.67±1.20 (25.57)	12.33±0.67 (20.54)	13.00±0.58 (21.12)	14.67 (22.41)
Mean	15.50 (21.94)	13.50 (20.58)	10.20 (16.98)	-	11.20 (18.74)	9.90 (17.28)	7.97 (14.18)	-	10.43 (18.16)	8.67 (16.33)	7.07 (13.25)	-
LSD (p\0.05)	Colony (2.28) Brood Cycle (1.25) Colony x Brood Cycle (3.94)				Colony (2.59) Brood Cycle (1.42) Colony x Brood Cycle (4.49)				Colony (2.59) Brood Cycle (1.42) Colony x Brood Cycle (4.49)			

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

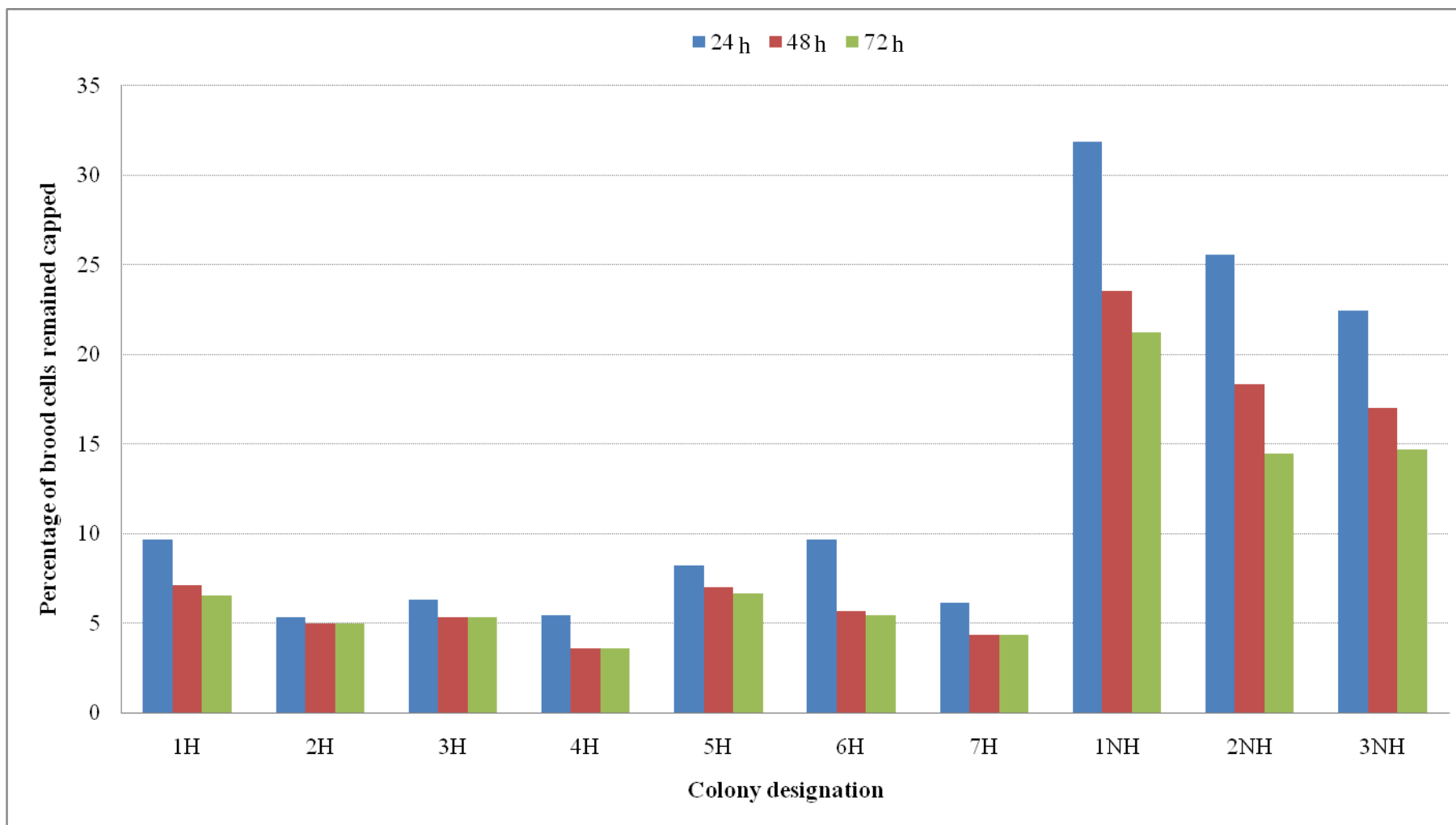


Fig. 7 Mean percentage of brood cells remained capped after 24, 48 and 72 h of pricking over the three alternate test brood cycles in the seven hygienic (1H-7H) and three non-hygienic (1NH-3NH) colonies

These findings also support the earlier findings of Palacio (2000) who evaluated the colonies for hygienic behaviour using a pin-killed brood assay and reported that hygienic behaviour in the colonies, headed by queens without mating control was 84.56 per cent. Bigio *et al* (2014) also observed that colonies headed by naturally-mated queens had high levels of hygiene. They reported that the dead brood removal in colonies headed with artificially inseminated hygienic queens ranged from 94.50-100 per cent and 57.80-100 per cent in the colonies with naturally mated queens. The present findings also corroborate with the findings of Nouredine and Heddad (2014). They conducted hygienic behaviour studies in 40 *A. mellifera intermissa* colonies of Algerian local bees in spring and fall and noted that the dead brood removal was 91.56 per cent in spring and 83.50 per cent in autumn. Joni and Mall (2017) also reported that after 24 hours, the mean dead brood removal in healthy colonies, was 37.57 per cent (range: 35.33-38.09%), while in infected colonies, it was 41.21 per cent (range 38.06- 43.15%). After 48 hours, in healthy colonies, mean was 90.39 per cent (range: 88.71- 92.08%) while in infected colonies, it was 98.28 per cent (range: 96.96-100.0%).

4.3 Molecular characterization of the bee colonies using SSR markers linked with hygienic behaviour

Ten adult worker bees were collected from each of the ten *A. mellifera* (see § 4.1.5) colonies headed by naturally mated queens (the 7 most hygienic and the 3 most non-hygienic) for each of the three test brood cycles at every six weeks interval between each of the two consecutive test brood cycles (300 bee samples) for genomic DNA isolation and were genotyped at the five SSR loci, reported to be linked with hygienic behaviour. K0263, A058 and K1601 were the nearest markers identified for the QTL *Hyg1*, *Hyg2* and *Hyg 3*, respectively. AT128 and K1601 were the nearest markers for the suggestive QTL responsible for uncapping component (*Uncap1* and *Uncap2*) and AC074 was the nearest marker for the removing component (*Rem1*). The banding profiles and the polymorphism produced by these markers are shown in Fig. 8, 9 and 10. Based on the PCR amplification profiles of the genomic DNA of the worker honey bees of all the 10 colonies with the above markers, it was observed that these markers did not discriminate between the hygienic and non-hygienic colonies, and all the five markers showed polymorphism. The allelic richness (average number of alleles per locus) of markers K0263, A058, K1601, AT128 and AC074 ranged between 2-4 and all the markers were 100 per cent polymorphic. The diversity index, resolving power, marker index and PIC (Polymorphic Information Content) of all the five markers ranged between 0.37-0.80, 2.39-6.15, 0.83-2.41 and 0.23-0.60, respectively. The SSR markers linked with hygienic behaviour could not differentiate between the hygienic and non-hygienic trait as the colonies were headed by the queens who might have undergone multiple matings (open natural mating) and the colony consisted of population representing sisters and

super sisters belonging to different patriline. Naturally mated queens yield quite different levels of intracolony genetic diversity, which affect overall colony performance.

4.4 Genetic diversity of *A. mellifera* colonies (seven hygienic and three non-hygienic) using SSR markers

4.4.1 SSR marker analysis of the adult honey bee worker genotypes

The genomic DNA extracted in § 4.3 was used for the present study. These 300 samples representing the 10 colonies were genotyped with 40 SSR markers to study the genetic diversity both within the colonies and among the colonies. Out of these 40 SSR markers, three were monomorphic and 37 were polymorphic. The amplification profiles and polymorphism generated by using the 37 markers are shown in Fig. 11, 12 and 13. These 37 markers produced a total of 137 scorable DNA fragments with an average of 3.7 fragments per marker of which 134 (97.81%) were polymorphic and three were monomorphic (2.19%). The number of alleles per marker ranged between two in markers UN368, A058, AC109 and K1610 and seven in markers SV198 and 5983, while three markers produced six alleles each, one marker amplified five alleles, 11 markers amplified four alleles each, and 16 markers produced three alleles each (Table 20). The size of the amplicons ranged between 80-450bp.

4.4.2 Polymorphic information content (PIC)

The PIC values, analyzed to characterize the capacity of each SSR markers to show polymorphic loci, ranged between 0.23 (K0263) to 0.77 (marker 5983). The average PIC value for all the evaluated loci was 0.52. Diversity index (DI)/ genetic diversity is the expected heterozygosity. The maximum diversity index was 0.92 for the marker SV198 and minimum of 0.37 was observed for the marker K0263. The mean DI value for all the evaluated markers was 0.68. Marker index (MI) is the capacity of each primer to detect polymorphism. The marker SV198 also recorded the maximum index value and the minimum was seen for the marker UN368 and the average MI value was 2.56. The Resolving power (Rp) ranged between 2.25 (B1293) and 6.30 (AP094) with a mean value of 3.40 (Table 20).

4.4.3 Genetic diversity

The dendrogram depicting the inter colony genetic diversity and relationship based on the SSR scoring data using weighted Neighbour Joining method was generated using the DARwin software version 6.0. The clustering pattern of the Neighbour joining dendrogram representing the 1H-7H hygienic colonies and 1NH-3NH non-hygienic colonies of the first brood cycle showed three clusters. Cluster I was further divided into two sub-clusters IA and IB and the whole cluster I comprised of four hygienic colonies viz., 7H, 6H, 5H and 2H. Cluster II and III consisted of both hygienic and non-hygienic colonies. Cluster II comprised of 2NH, 1H, 4H and 1NH genotypes and the cluster III consisted of 3H and 3NH (Table 21, Fig 14).

Table 20. Total No. of alleles, Diversity index, Resolving power, Marker index and PIC values of SSR primers

Marker	Total no. of alleles	Diversity Index (DI)	Resolving power (Rp)	Marker Index (MI)	Polymorphism information content (PIC)
K0206	5	0.50	6.17	2.50	0.38
K0264	4	0.80	2.45	3.20	0.69
AP098	4	0.71	3.70	2.83	0.54
K0263	4	0.37	6.15	0.83	0.23
UN368	2	0.48	2.37	0.24	0.41
SV198	7	0.92	3.58	6.41	0.74
BI036	3	0.52	3.67	1.55	0.39
SV172	3	0.69	3.28	2.08	0.45
A058	2	0.61	2.48	1.21	0.38
K0519	6	0.83	3.83	4.96	0.68
5983	7	0.85	3.29	5.93	0.77
K0520	4	0.69	3.86	2.75	0.52
K0521	6	0.74	4.87	4.41	0.59
AC011	3	0.72	2.27	2.17	0.62
AT128	4	0.78	3.58	3.13	0.55
BI293	3	0.74	2.25	2.21	0.57
SV026	3	0.65	2.64	1.94	0.56
AG016	3	0.72	2.86	2.15	0.52
K0902	4	0.74	3.31	2.96	0.59
5242	4	0.66	4.27	2.63	0.47
K1076	4	0.81	3.41	3.24	0.57
K1074	4	0.74	3.91	2.97	0.51
AC074	3	0.80	2.47	2.41	0.59
AP146	4	0.84	2.78	3.37	0.65
AC109	2	0.45	2.88	0.90	0.28
AC138	4	0.73	3.95	2.94	0.51
BI119	3	0.56	3.61	1.69	0.40
K1012	3	0.72	2.62	2.17	0.56
BI324	3	0.68	2.80	2.03	0.53
K1601	3	0.68	2.39	2.05	0.60
AP217	3	0.72	2.99	2.17	0.50
AP028	3	0.74	3.08	2.21	0.49
K1610	2	0.47	2.51	0.95	0.37
AP094	6	0.57	6.30	3.43	0.55
K1638	3	0.65	3.09	1.94	0.49
6560	3	0.70	3.01	2.09	0.50
K1656	3	0.73	3.09	2.20	0.48
Mean	3.70	0.68	3.40	2.56	0.52

A058

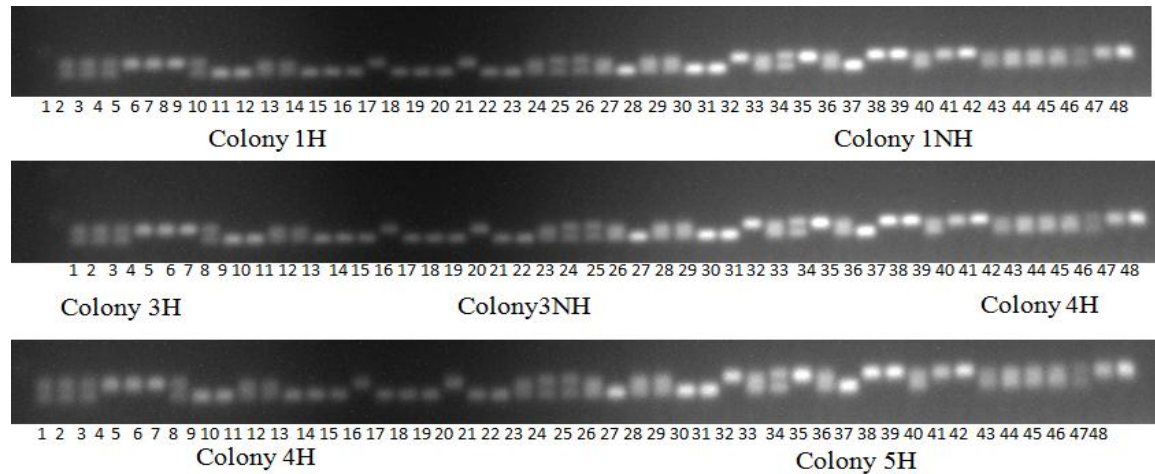
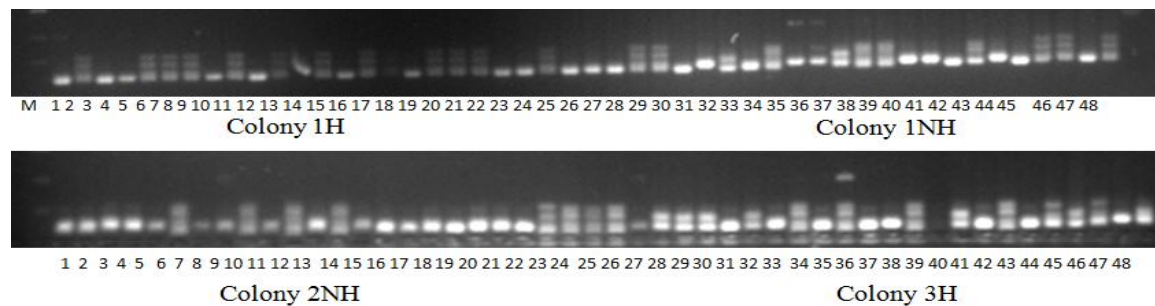


Fig. 8 Gel electrophoresis profiles of PCR amplified DNA of hygienic and non-hygienic colonies with SSR marker A058. Lanes 1-30 Colony 1H; Lanes 31-48 Colony1 NH; Lanes 25-48 3H& 1-6 3H; Lanes 7-36 Colony 3NH; Lanes 1-12 and 1-18 Colony 4H; Lanes 19-48 Colony 5H

AT128



AC074

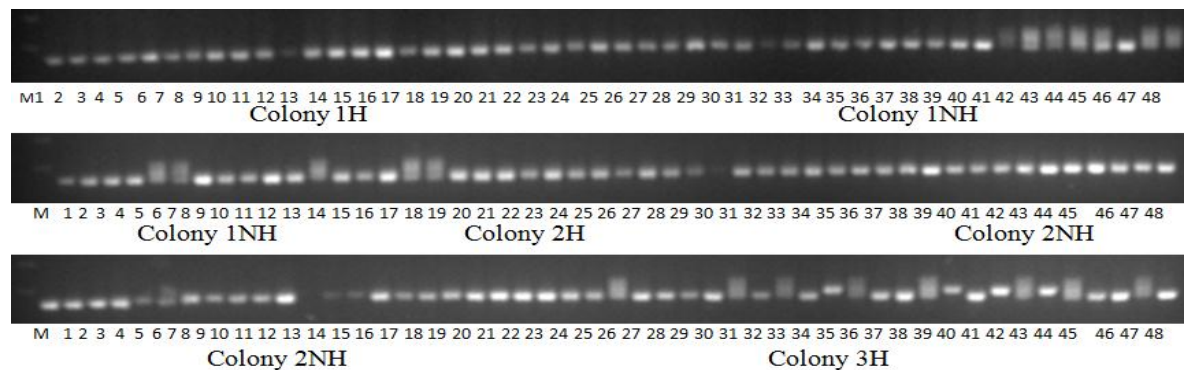


Fig. 9 Gel electrophoresis profiles of PCR amplified DNA of hygienic and non-hygienic colonies with SSR marker AT128 & AC074. Lanes 1-30 Colony 1H; Lanes 31-48 & 1-12 Colony1 NH; Lanes 13-42 Colony 2H; Lanes 43-48 & 1-24 Colony 2NH; Lanes 25-48 3H

K1601

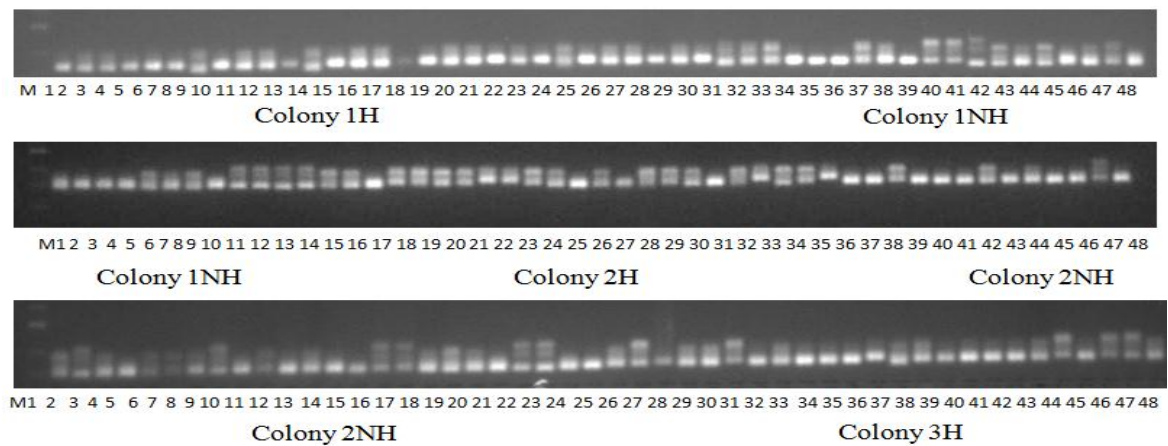
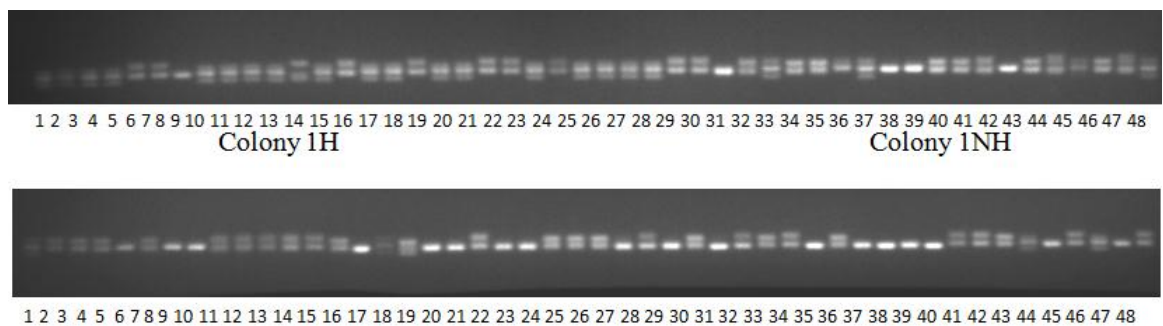


Fig. 10 Gel electrophoresis profiles of PCR amplified DNA of hygienic and non-hygienic colonies with SSR marker K1601. Lanes 1-30 Colony 1H; Lanes 31-48 & 1-12 Colony1 NH; Lanes 13-42 Colony 2H; Lanes 43-48 & 1-24 Colony 2NH; Lanes 25-48 3H

AP098



SV198

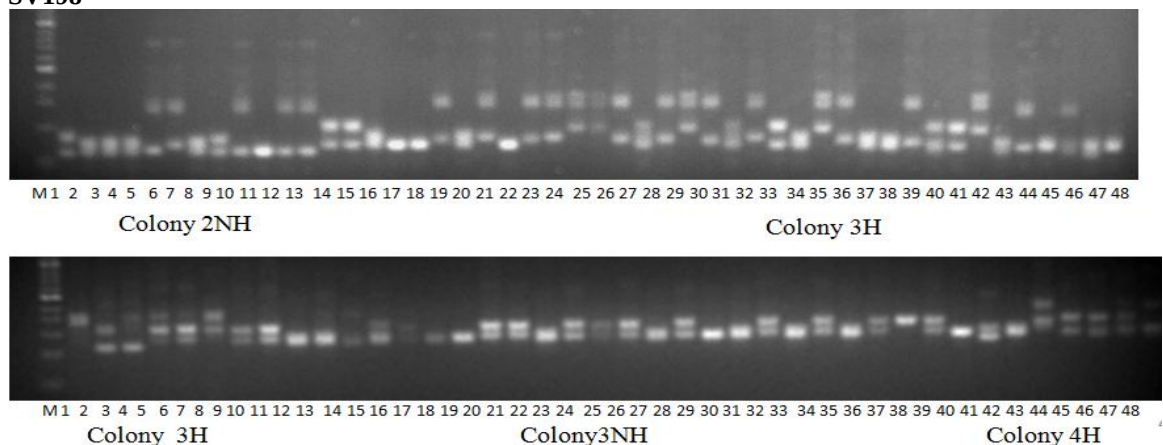
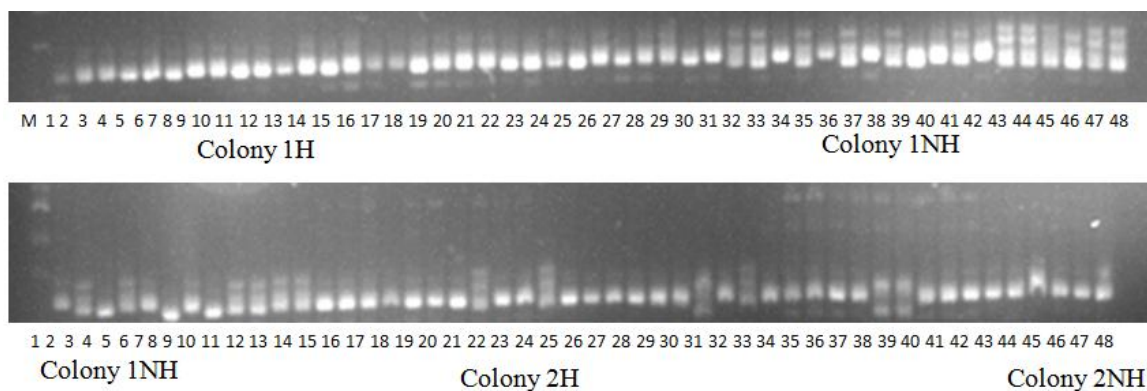


Fig. 11 Gel electrophoresis profiles of PCR amplified DNA of hygienic and non-hygienic colonies with SSR marker AP098. Lanes 1-30 Colony 1H; Lanes 31-48 & 1-12 Colony1 NH; Lanes 13-42 Colony 2H; Lanes 43-48 Colony 2NH; SV198: Lane M 50bp DNA Marker; Lanes 1-30 Colony 2NH; Lanes 31-48 & 1-12 Colony3H; Lanes 13-42 Colony 3NH; Lanes 43-48 Colony 4H

K0519



K0520

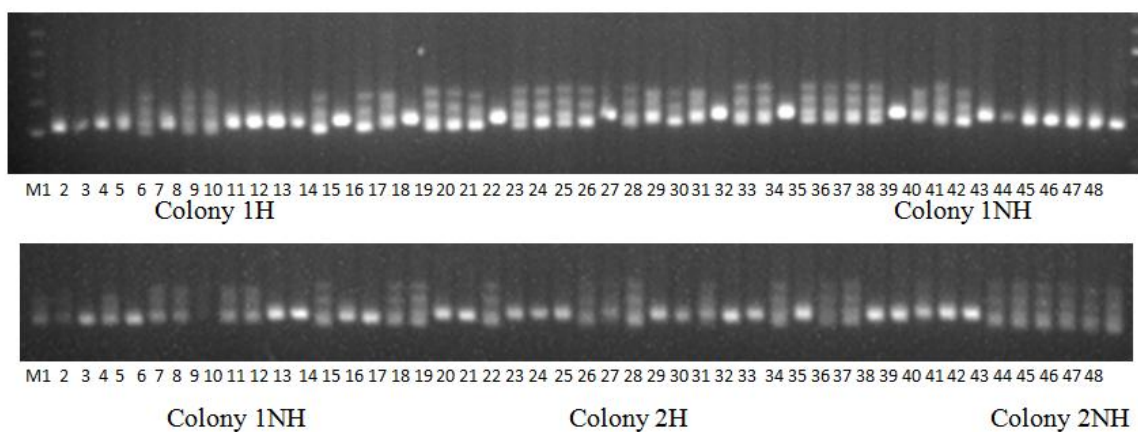
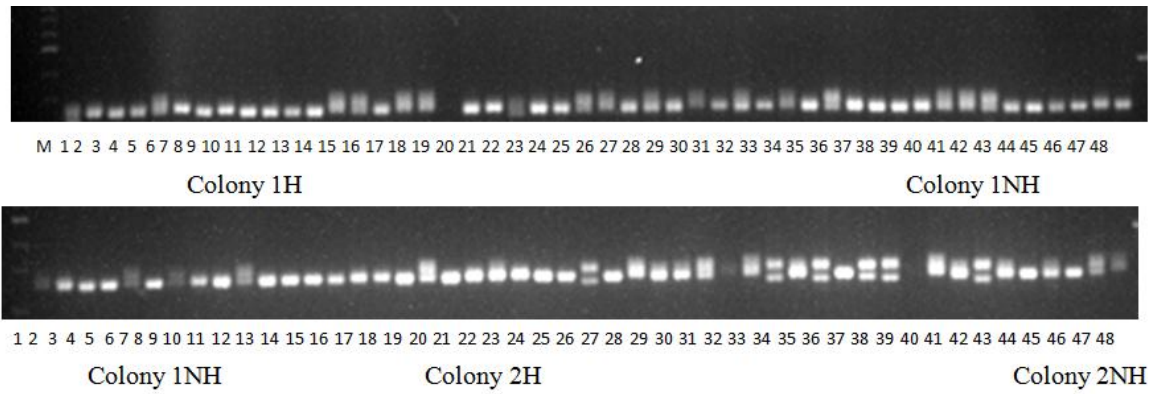


Fig. 12 Gel electrophoresis profiles of PCR amplified DNA of hygienic and non hygienic colonies with SSR markers K0519 &K0520. Lane M 50bp DNA Marker; Lanes 1-30 Colony 1H; Lanes 31-48 & 1-12 Colony1 NH; Lanes 13-42 Colony 2H; Lanes 43-48 Colony 2NH;

B1293



S242

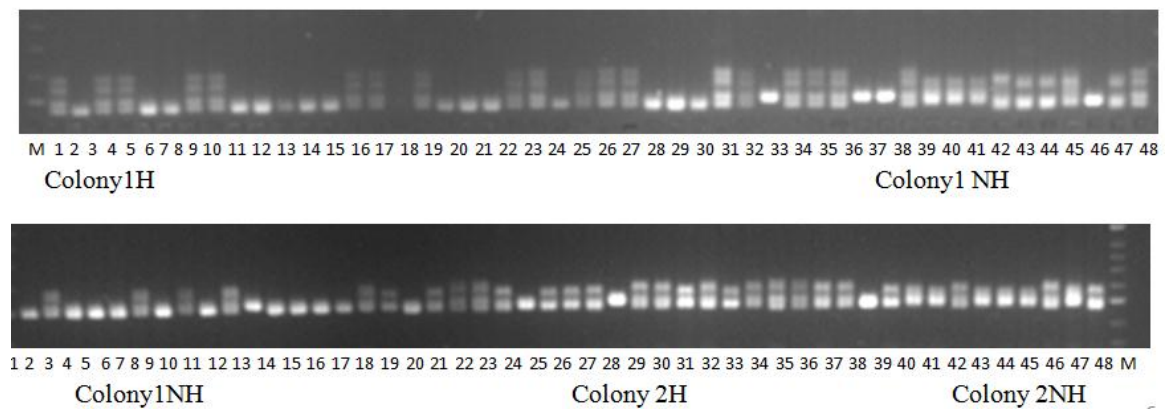


Fig. 13 Gel electrophoresis profiles of PCR amplified DNA of hygienic and non-hygienic colonies with SSR markers B1293 & 5242. Lane M 50bp DNA Marker; Lanes 1-30 Colony 1H; Lanes 31-48 & 1-12 Colony1 NH; Lanes 13-42 Colony 2H; Lanes 43-48 Colony 2NH

The dendrogram of the second test brood cycle at six weeks interval too comprised of three clusters (Table 22, Fig 15). Cluster I was the largest consisting of five hygienic (6H, 7H, 4H, 1H, 2H) and one non-hygienic colony (1NH) and also a small population belonging to colony 3H. The Cluster II consisted of 3NH and 3H colonies. The cluster III consisted of 5H and 2NH colonies.

The clustering pattern of the genotypes of the third test brood cycle sampled at six weeks, have also showed three clusters. Cluster I (3NH, 2H, 1H and 5H) and cluster II (3H, 2NH, 1NH and 4H) consisted of four colonies each, both of hygienic and non-hygienic traits, and the cluster III comprised of colonies 6H and 7H, both consistently forming the same group among all the brood cycles though varying in cluster positions (Table 23, Fig 16).

The dendrogram of 300 genotypes representing the ten colonies over the three test brood cycles, neighbour joining tree was generated showing the clustering pattern of 1H-7H hygienic colonies and 1NH-3NH non-hygienic colonies. Similar to the earlier studied three individual brood cycles as above, the dendrogram comprised of three clusters I, II and III. Cluster I comprised of four hygienic colonies 7H, 6H, 1H and 2H. The colonies 5H, 2NH, 4H, 3H, and 1NH made up the cluster II, and the cluster III consisted of a single non-hygienic 3NH colony. The grouping pattern of the seven hygienic and three non-hygienic colonies varied in all the dendrograms constructed for the three individual test brood cycles and also in the dendrogram of all the 300 genotypes except for the colonies 6H and 7H which consistently formed one group, though present in varied clusters (Table 24, Fig. 17).

The similarity coefficient based on genomic DNA amplification of the samples of honey bee workers using microsatellite markers was estimated by dice similarity coefficient. The similarity coefficient of colonies in consecutive first, second and third test brood cycles sampled at six weeks interval ranged between 0.49-0.97, 0.46-0.97 and 0.50-0.97, respectively. Overall, in the colonies across all the three test brood cycles, it ranged between 0.47-.98 with a mean value of 0.72.

Hence, SSR markers used in the present study could generate genetic diversity between the colonies. Thus, these SSR markers can be used in future for genetic diversity studies.

The present findings are in corroboration with the studies conducted by Munoz *et al* (2008) where three types of honey bee pupae: pupae not parasitized, infested pupae with mites without reproductive success and infested pupae with mites with reproductive success, were genotyped with five microsatellite loci and analysis revealed that different groups of pupae were differentiated by a different allelic composition. In the principal coordinate analysis, two main clusters, cluster I consisted of 95.90 per cent non-infested pupae and 68.80 per cent infested pupae with mites without reproductive success and cluster II comprised of 94.1 per cent infested pupae with mites with reproductive success and 31.20 per cent infested pupae with mites without reproductive success.

Table 21. Clustering pattern and similarity coefficients of colonies in brood cycle I

Cluster	Groups	Colonies	Similarity Coefficients
I	Ia	7H, 6H, 5H	0.49-0.97
	Ib	2H	
II	IIa	2NH,1H, 4H	
	IIb	1NH	
III	IIIa	3H	
	IIIb	3NH	

Table 22. Clustering pattern and similarity coefficients of colonies in brood cycle II

Cluster	Groups	Colonies	Similarity Coefficients
I	Ia	6H,7H,4H,1NH, 3H	0.46-0.97
	Ib	1H,2H	
II	IIa	3NH	
	IIb	3H	
III	IIIa	5H	
	IIIb	2NH	

Table 23. Clustering pattern and similarity coefficients of colonies in brood cycle III

Cluster	Groups	Colonies	Similarity Coefficients
I	Ia	3NH,2H,1H	0.50-0.97
	Ib	5H	
II	IIa	3H,2NH,1NH	
	IIb	4H	
III	IIIa	6H	
	IIIb	7H	

Table 24. Clustering pattern and similarity coefficients of colonies across all the three test brood cycles

Cluster	Groups	Colonies	Similarity Coefficients
I	Ia	7H,6H	0.47-0.98
	Ib	1H,2H	
II	IIa	5H,2NH	
	IIb	4H,3H,1NH	
III		3NH	

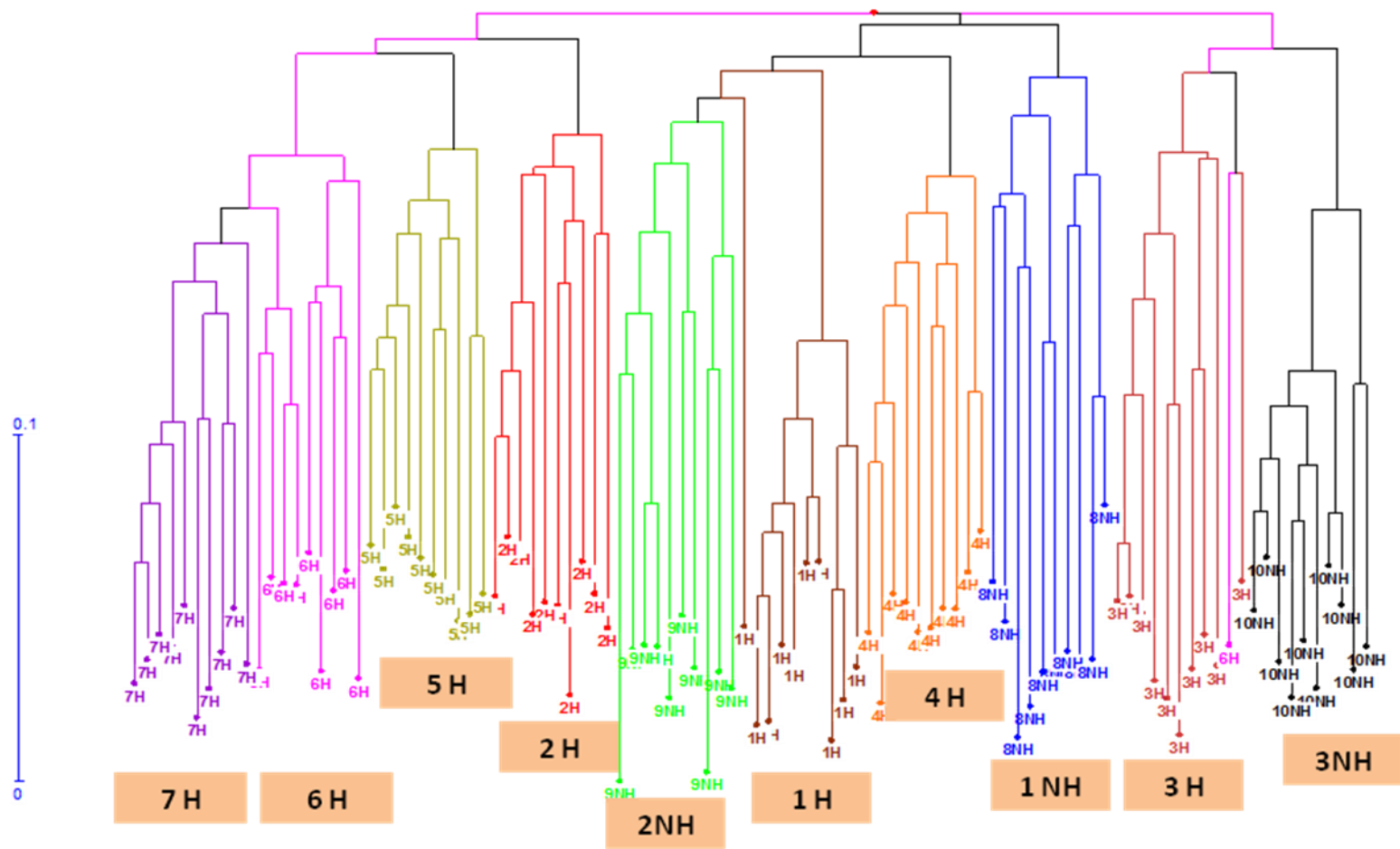


Fig. 14 Neighbour joining dendrogram showing clustering pattern of 1H-7H hygienic colonies and 1NH- 3NH non-hygienic colonies in the first brood cycle

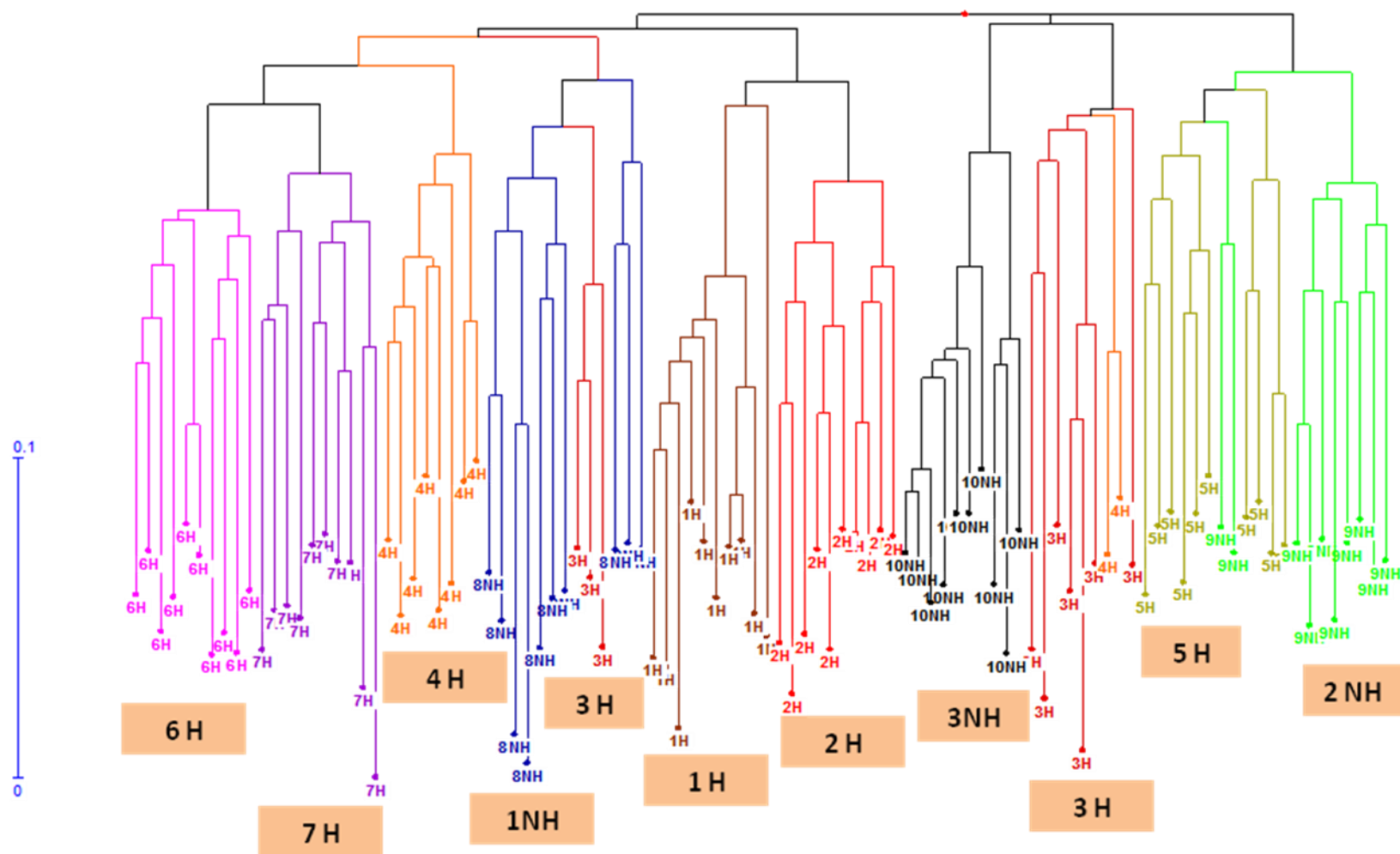


Fig. 15 Neighbour joining dendrogram showing clustering pattern of 1H-7H hygienic colonies and 1NH-3NH non-hygienic colonies in the 2nd alternate test brood cycle at six weeks interval

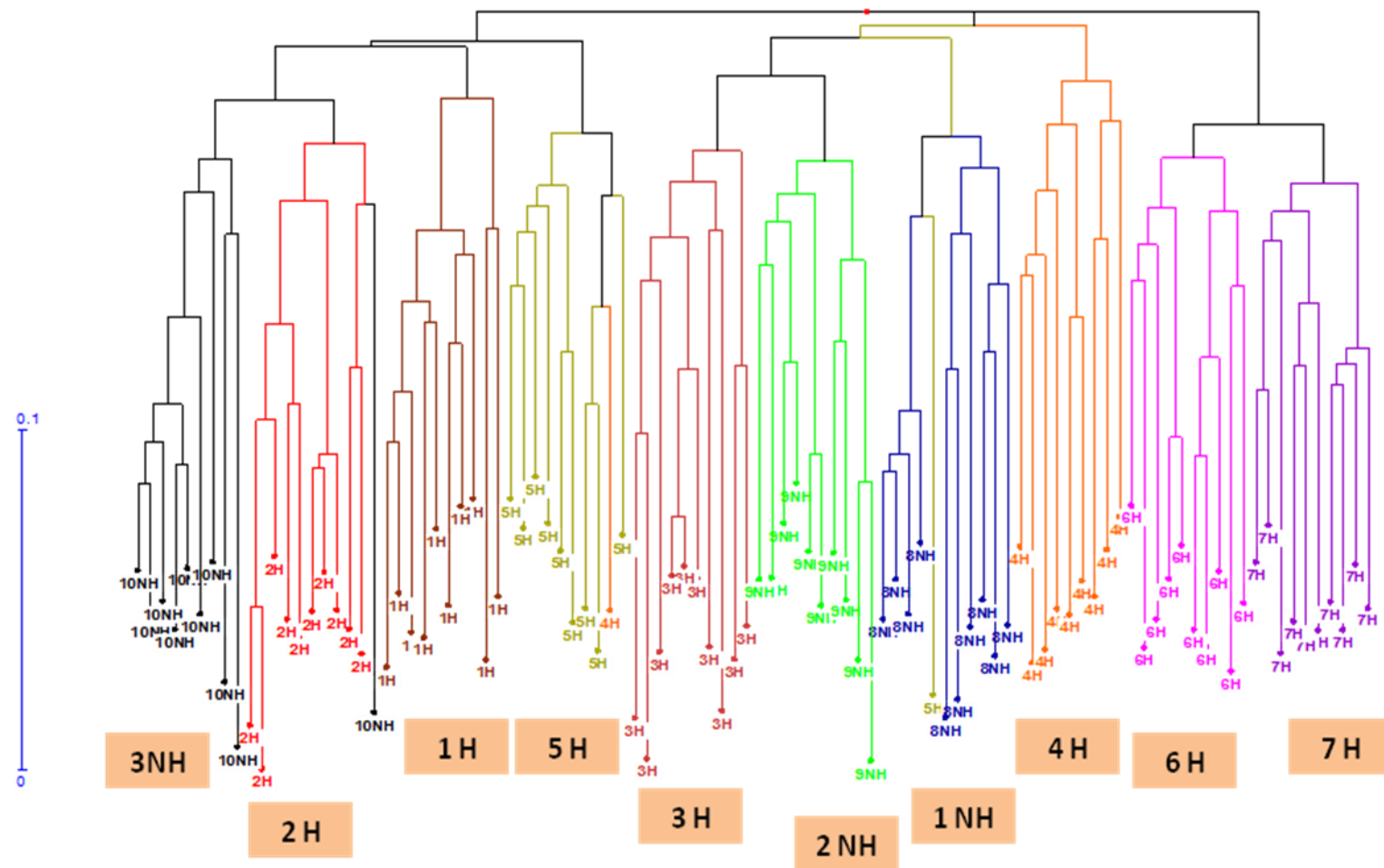


Fig. 16 Neighbour joining dendrogram showing clustering pattern of 1H-7H hygienic colonies and 1NH-3NH non-hygienic colonies in the 3rd alternate test brood cycle at six weeks interval

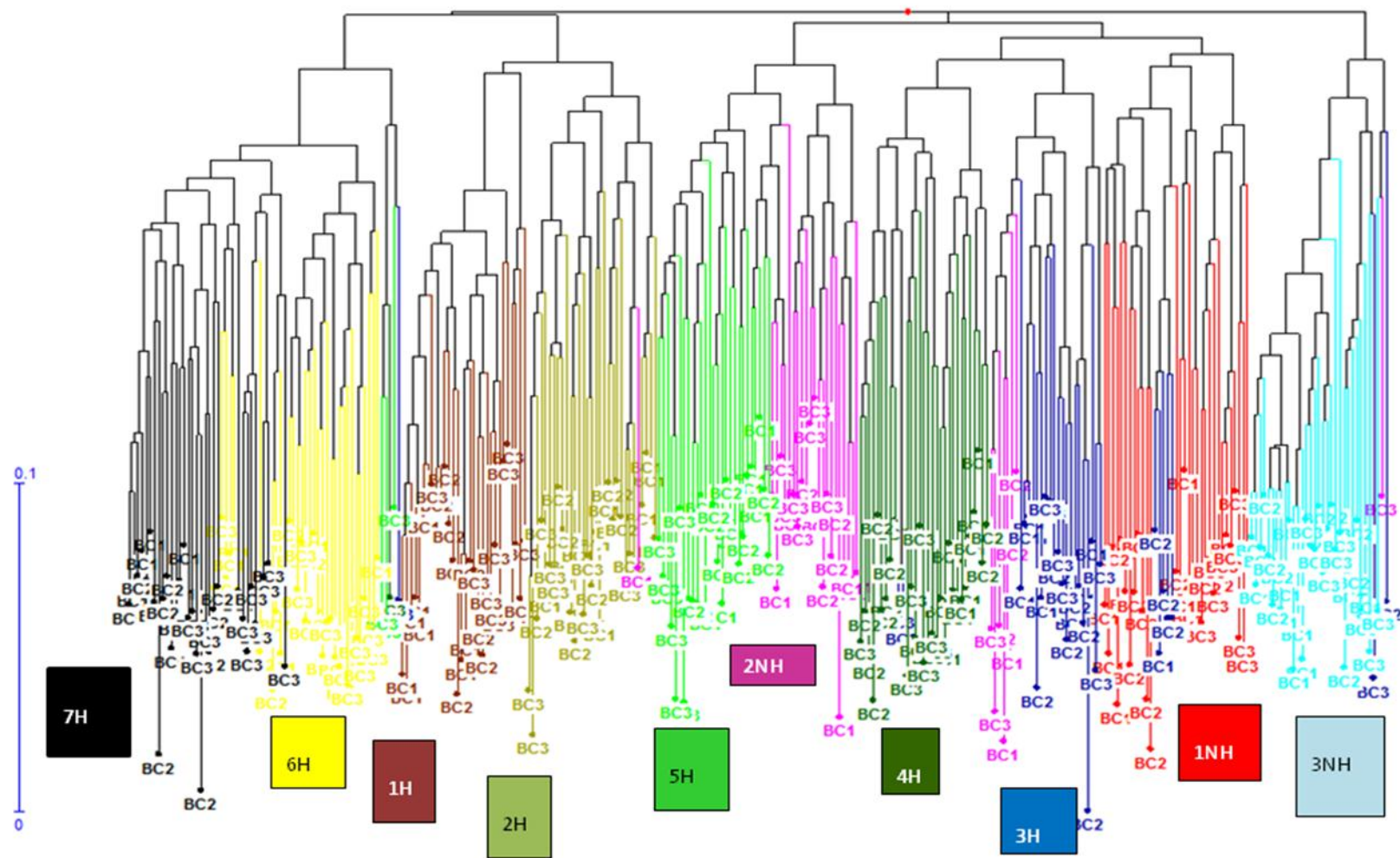


Fig.17 Neighbour joining dendrogram showing clustering pattern of 1H-7H hygienic colonies and 1NH- 3NH non-hygienic colonies in all the three alternate brood cycles

Irene and de La Rua (2012) conducted temporal variation studies and observed no significant differences in heterozygosity or allelic frequency between the two surveys conducted in 1998 and 2006. In 1998, the number of alleles per *locus* ranged from two- seven, whereas they varied from three-seven in 2006. The mean heterozygosity values were 0.437 in 1998 and 0.454 in 2006. The mean number of alleles per *locus* (4.0) and the total number of alleles (20) did not vary between 1998 and 2006.

Honey bee colonies are made up of a large number of patriline (~10-30), each patriline comprised of highly related super sisters headed by the same father. Differences among patriline show variation in the frequency of task performance and explain that among patriline, a genetic component is responsible for varying task thresholds (Robinson1992, Robinson and Huang 1998). The importation of foreign queens and the practice of migrating colonies several times per year can affect the genetic structure of a local honey bee population through genetic introgression (Garnery *et al* 1998). Determination of genetic variation of honey bee populations using different markers will provide a valuable resource for selection for desired traits that may be tolerant to disease causing agents and ectoparasites and for conservation purposes.

Tunca and Kence (2011) used 20 Random Amplified Polymorphic DNA (RAPD) primers to assess the genetic diversity in 720 worker bees collected from 360 colonies of 25 provinces in Turkey. Ten primers produced 105 reproducible bands, all polymorphic. Mean genetic diversity values ranged between 0.035 and 0.175.

Munoz *et al* 2009 reported genetic diversity between 9.25 and 9.75 alleles per locus in indigenous populations of *A. m. carnica* and/or *ligustica* in Croatia and Italy. In indigenous Italian and Croatian population heterozygosity was 0.638 and 0.674, respectively, whereas in US commercial populations Delaney *et al* (2009) reported it to be 0.53. Angelique *et al* (2015) studied a total of 524 *A mellifera* colonies from 16 beekeepers in 20 sites in Rodrigues, an island in the South-West Indian Ocean with 18 microsatellite markers. The mean number of alleles over all the sites was 7.63 per locus (range: 3.00-15.00) and heterozygosity was ($H_{obs}=0.644$).

Further the selective breeding programs can enhance the levels of hygienic and provide significant resistance to diseases and parasites. Colony level genetic diversity plays a major role in both disease resistance and division of labor, resulting in greater productivity and colony strength.

4.5 Molecular characterization of colonies contrasting in hygienic behaviour with the primers designed from the candidate genes for hygienic behaviour

4.5.1 By the presence/ absence of candidate genes for hygienic traits

The same genomic DNA extracted from a total of 300 samples of adult worker honey bees (10 each from the seven hygienic colonies and the three non-hygienic colonies for each of the three consecutive test brood cycles i.e. sampled at six weeks intervals) were used to amplify the candidate genes for hygienic behaviour with the gene specific primers. Seventeen candidate genes for hygienic behaviour were selected to study if they varied among the hygienic and non-hygienic colonies. Two primers per candidate gene were used to amplify genomic DNA of 10 colonies. It was observed that all the newly designed primers amplified all the 300 individuals, generating amplicons of 450-550 bp and no length polymorphism between the amplicons of hygienic and non-hygienic traits and intra colony variation were observed.

4.5.2 By sequencing the candidate genes for hygienic traits

Among the 17 candidate genes studied w.r.t § 4.5.1, five genes were selected, viz., *GB13839*, *GB12228*, *GB12301*, *GB11135* and *GB19509*. Four DNA samples each were taken from one most hygienic colony and from one most non-hygienic colony. The DNA samples were PCR amplified with the primers of the five selected candidate genes. A small volume of 5 µl of the PCR product was resolved on 1.0 per cent agarose gel to confirm the amplification of the desired gene before sending for custom sequencing services.

The genes, *GB13839*, *GB12228* (Fig. 18), showed no sequence variation, thus showing that these genes are totally conserved genes. The gene *GB12301* (Fig 19) showed sequence differences at several sites, but no variation was found between the hygienic and non-hygienic colonies. The gene *GB19509* (Fig 20) associated with olfactory behaviour; showed sequence differences at several sites indicating genetic differences at multiple sites. Variations were found at several sites and also between hygienic and non-hygienic trait. At 282 position, Adenine occurs in the hygienic samples and Thymine in the non-hygienic samples; at position 309, Guanine is in the hygienic colony samples and Adenine in the non-hygienic samples; at 358, Guanine occurs in the hygienic samples and Thymine in the non-hygienic samples; at position 398, Cytosine is in the hygienic colony and Thymine in the non-hygienic colony; at 410, Cytosine in the hygienic colony and Adenine occurs in the non-hygienic colony; Adenine occurs in the hygienic samples and Cytosine occurs in the non-hygienic samples at a position of 460. Thus, in this gene, six differential SNPs were observed between hygienic and non-hygienic colonies. The present study suggests the presence of genetic variation which might be related to hygienic trait in honey bees. Cornelissen (2015) reported the presence of genetic variation in *Dop3* and *Prohormone-1*, which might be related to *Varroa* resistance in honey bees.

GB12228 Transcription coactivator activity

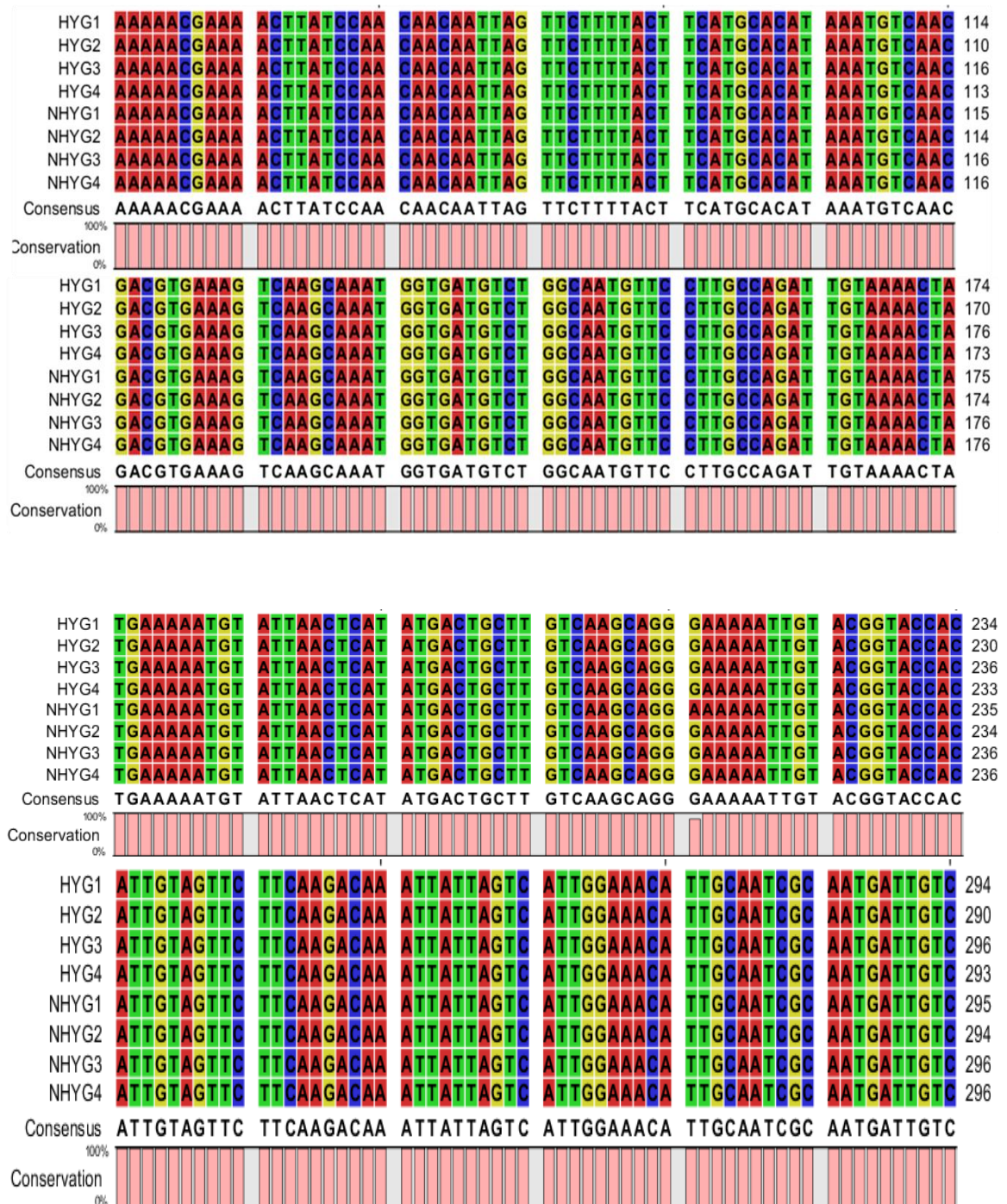


Fig. 18 Multiple sequence alignment of 4 each sequences representing hygienic and 4 non-hygienic of geneGB 12228 using software program CLC workbench version 7.5

GB12301 Social behaviour;transcription activator activity

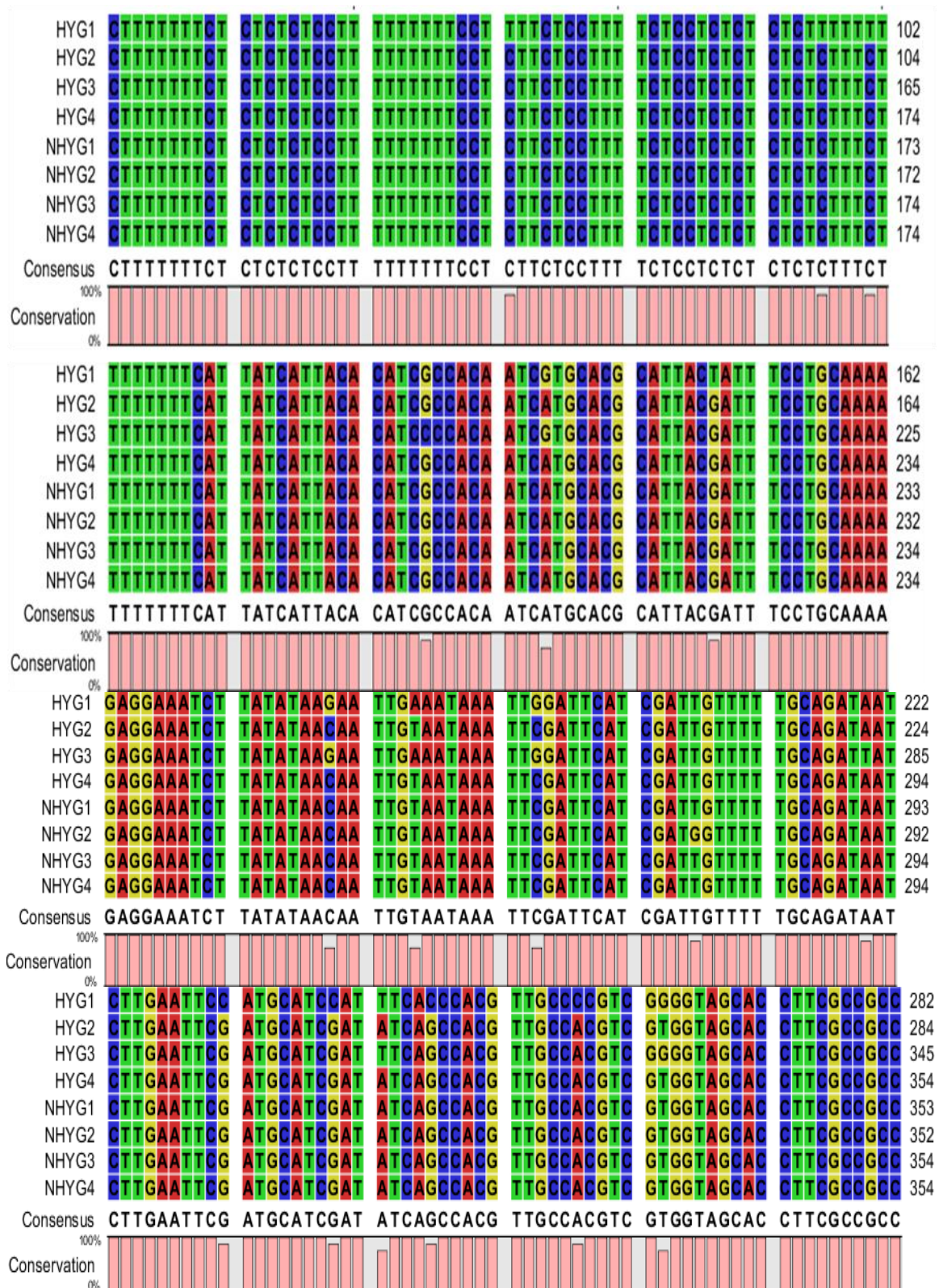


Fig. 19 Multiple sequence alignment of 4 each sequences representing hygienic and 4 non-hygienic of geneGB 12301 using software program CLC workbench version 7.5

GB19509 Olfactory behaviour

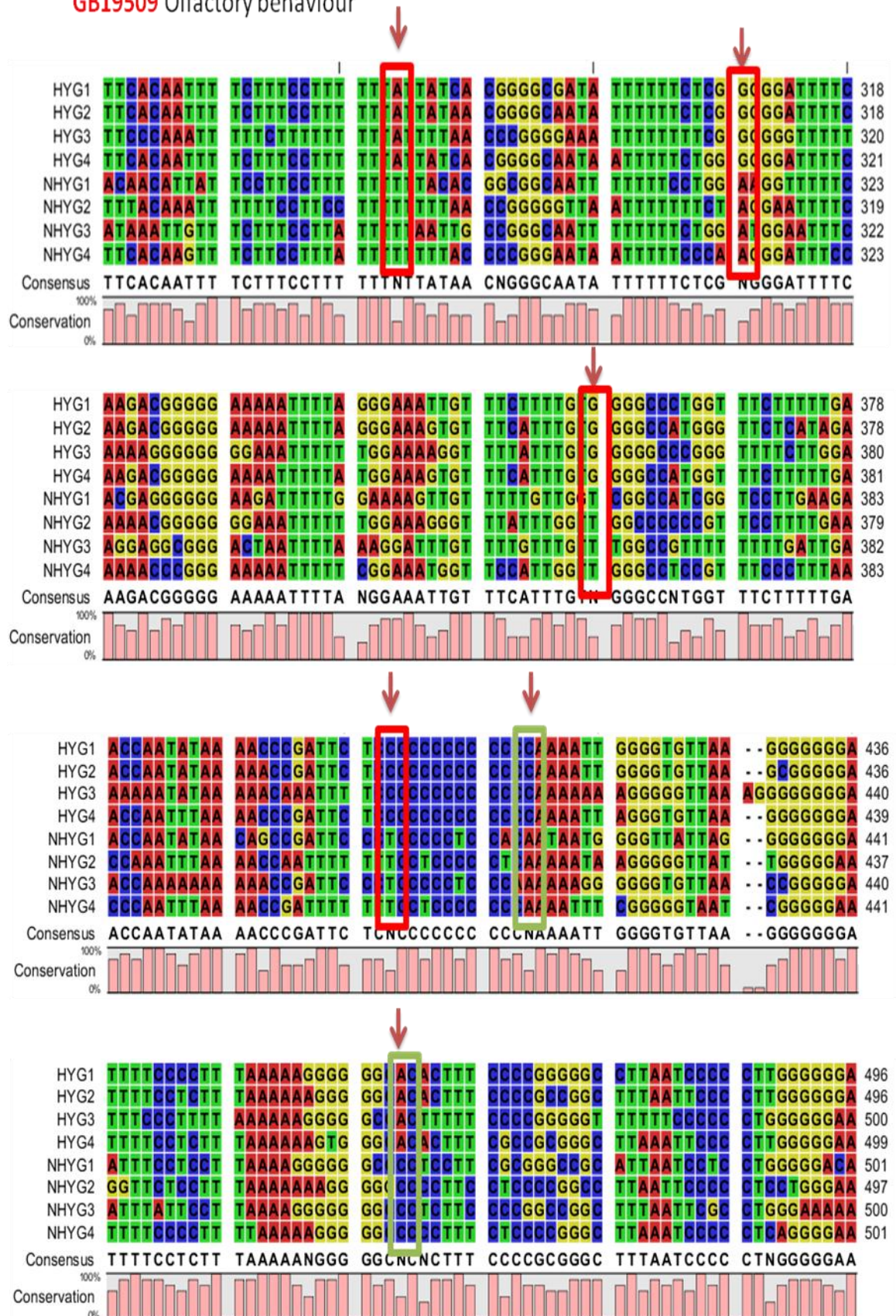


Fig. 20 Multiple sequence alignment of 4 each sequences representing hygienic and 4 non-hygienic of geneGB 19509 using software program CLC workbench version 7.5

4.6 Temporal variation in hygienic behaviour response among the selected hygienic and non-hygienic colonies

The colony level hygienic behaviour of the selected 10 colonies was assessed at every 2 h interval after treatment till the maximum of the pricked brood cells were emptied.

4.6.1 Dead brood removal after various time intervals of pricking in three alternate brood cycles

It was observed that within 2 h of the pricking of brood cells, except for hygienic colonies in each of the test brood cycle, no cleaning of brood cells was done. The mean percentage of dead brood removal in all the colonies was negligible as it ranged between 0 to 1.11 per cent. After 4h of treatment in first test brood cycle, it was in the range of 3 to 12.00 per cent in hygienic colonies and 0.67 to 2.67 per cent in non-hygienic colonies. In the second and third alternate test brood cycle, the dead brood cells emptied ranged between 1.00-8.68 and 4.33-9.67 per cent in hygienic colonies and 0.00-1.33 and 6.00-6.33 per cent in non-hygienic colonies, respectively. After 6 h, in each of the three brood cycles, the brood cells emptied ranged between 15.33-20.67;12.00-20.00 and 15.00-21.00 per cent in the 7 hygienic colonies and 5.00-7.67;4.67-7.00 and 9.33-13.67 per cent in the non-hygienic colonies, respectively. The mean of all the three brood cycles was 17.35 and 8.00 per cent in hygienic and non- hygienic colonies, respectively (Table 25, Fig. 21 and 22).

On the next day, i.e. after (Table 26, Fig. 21 and 22) 20 h of the pricking, in the hygienic colonies, in the three alternate test brood cycles, the dead brood cells emptied were in the range of 76.00-94.00 per cent, 81.00-92.00 and 82.00-93.67 per cent, respectively. Thus, except for the colonies in the first brood cycle, in the remaining two brood cycles all the colonies showed hygienic behaviour. In the non-hygienic colonies in the three consecutive brood cycles, the hygienic behaviour was in the range of 37.33-48.33, 27.33-47.00 and 35.00-39.33 per cent, respectively. The overall mean of the pricked brood cells emptied was 86.97 per cent for the hygienic colonies and 39.6 per cent for the non-hygienic colonies. After 22 h of treatment, the frequency of hygienic behaviour increased in all the colonies and the hygienic designated colonies during the three consecutive test brood cycles recorded hygienic trait response (complete removal of dead brood cells) in the range of 81.67-95.00, 84.00-94.00 and 87.33-96.33 per cent, respectively. The corresponding figures in the non-hygienic colonies were 44.00-50.67, 38.33-51.00 and 40-41.00 per cent. In the hygienic and non-hygienic colonies, the mean of 90.13 and 44.15 per cent of the pricked brood cells were emptied. At 24 h, the dead brood cells emptied in each of the three brood cycles was in the range of 82.00-95.00, 82.67-94.67 and 91.33-96.67 per cent in the seven hygienic colonies, respectively; while in the non-hygienic colonies, it was in the range of 45.67-55.33, 40.67-54.00 and 44.33-48.00, respectively.

Table 25. Percentage of brood cells emptied by various time intervals after pricking in three alternate brood cycles*

Col. No.	2 h				4 h				6 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	0.00±0.00 (0.00)	2.67±1.76 (7.43)	0.67±0.67 (2.71)	1.11±0.80 (3.38)	12.00±0.58 (20.25)	7.00±0.58 (15.32)	7.67±0.33 (16.06)	8.89±1.57 (17.21)	19.00±0.58 (25.83)	15.67±1.45 (23.26)	16.00±0.58 (23.56)	16.89±1.06 (24.22)
2H	0.00±0.00 (0.00)	0.00±0.00 (0.00)	1.00±0.58 (4.62)	0.33±0.33 (1.54)	9.67±0.33 (18.10)	1.00±1.00 (3.32)	9.67±0.33 (18.10)	6.78±2.89 (13.18)	20.67±0.67 (27.02)	16.67±0.88 (24.07)	21.00±0.00 (27.26)	19.44±1.39 (26.12)
3H	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	8.67±0.67 (17.09)	8.67±0.67 (17.09)	9.00±0.00 (17.45)	8.78±0.11 (17.11)	18.67±1.20 (25.57)	20.00±1.15 (26.54)	19.67±0.33 (26.31)	19.44±0.40 (26.14)
4H	0.67±0.67 (2.71)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.22±0.22 (0.90)	3.00±0.58 (9.88)	1.67±0.88 (6.03)	4.67±0.33 (12.45)	3.11±0.87 (9.45)	15.33±0.88 (23.03)	15.00±0.00 (22.78)	16.00±0.58 (23.56)	15.44±0.29 (23.12)
5H	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	4.33±0.67 (11.93)	2.67±1.33 (7.69)	5.00±0.58 (12.87)	4.00±0.69 (10.83)	17.33±0.33 (24.59)	14.67±0.33 (22.51)	15.00±0.58 (22.77)	15.67±0.84 (23.29)
6H	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	3.33±0.88 (10.34)	3.00±0.58 (9.88)	4.33±0.33 (11.99)	3.56±0.40 (10.74)	19.33±0.33 (26.07)	17.67±1.45 (24.81)	20.33±0.33 (26.79)	19.11±0.78 (25.89)
7H	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	6.67±0.67 (14.92)	4.67±1.33 (12.16)	6.33±0.33 (14.56)	5.89±0.62 (13.88)	17.33±1.20 (24.57)	12.00±0.00 (20.26)	17.00±0.58 (24.33)	15.44±1.72 (23.05)
1NH	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	2.67±1.33 (7.69)	1.33±1.33 (7.69)	6.33±0.88 (14.50)	3.44±1.49 (8.68)	6.67±0.33 (14.95)	4.67±1.67 (12.12)	9.33±0.33 (17.78)	6.89±1.35 (14.95)
2NH	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.67±0.67 (2.71)	0.00±0.00 (0.00)	6.00±0.58 (14.14)	2.22±1.90 (5.62)	7.67±0.67 (16.04)	7.00±1.00 (15.25)	13.67±0.88 (21.67)	9.44±2.12 (17.65)
3NH	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	1.33±0.67 (5.42)	0.00±0.00 (0.00)	6.33±0.33 (14.56)	2.56±1.93 (6.66)	5.00±0.58 (12.87)	7.00±0.58 (15.31)	11.00±0.58 (19.35)	7.67±1.76 (15.84)
Mean	0.07 (0.27)	0.27 (0.74)	0.17 (0.73)	-	5.23 (11.83)	3.00 (7.53)	6.53 (14.67)	-	14.70 (22.05)	13.03 (20.69)	15.90 (23.34)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

	<u>2h</u>	<u>4h</u>	<u>6h</u>
Colony	(1.82)	(2.89)	(1.19)
Brood Cycle	NS	(1.58)	(0.65)
Colony x Brood Cycle	(3.16)	(5.00)	(2.06)

Table 26. Percentage of brood cells emptied by various time intervals after pricking in three alternate brood cycles* starting at 20 h of treatment

Col. No.	20 h				22 h				24 h			
	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean
1H	90.33±0.33 (71.86)	90.00±0.00 (71.54)	90.33±0.33 (71.86)	90.22±0.11 (71.75)	92.33±0.33 (73.90)	92.33±0.33 (73.90)	91.67±0.33 (73.20)	92.11±0.22 (73.67)	94.33±0.33 (76.21)	94.67±0.33 (76.63)	94.00±0.00 (75.79)	94.33±0.19 (76.21)
2H	93.33±1.67 (75.21)	91.67±0.67 (73.22)	93.00±0.58 (74.65)	92.67±0.49 (74.36)	94.00±0.58 (75.88)	92.33±0.33 (73.90)	93.67±0.33 (75.40)	93.33±0.51 (75.06)	95.00±0.58 (77.09)	94.33±0.33 (76.21)	94.33±0.33 (76.21)	94.56±0.22 (76.50)
3H	94.00±0.58 (75.82)	92.00±0.00 (73.54)	93.67±0.33 (75.40)	93.22±0.62 (74.92)	95.00±0.33 (77.09)	94.00±0.58 (75.82)	95.67±0.33 (77.97)	94.89±0.49 (76.96)	95.00±0.58 (77.09)	94.00±0.58 (75.82)	95.67±0.33 (77.97)	94.89±0.49 (76.96)
4H	80.00±1.15 (63.43)	81.00±0.00 (64.13)	84.67±0.67 (66.93)	81.89±1.43 (64.83)	80.00±1.15 (63.43)	85.00±0.00 (67.19)	87.33±0.88 (69.15)	84.11±2.16 (66.59)	82.00±1.15 (64.89)	89.67±1.67 (71.35)	92.00±1.00 (73.61)	87.89±3.02 (69.95)
5H	76.00±1.00 (60.65)	84.67±1.45 (66.96)	82.00±1.15 (64.89)	80.89±2.57 (64.17)	81.67±1.00 (64.65)	86.67±1.45 (68.58)	88.33±1.15 (70.05)	85.56±2.00 (67.76)	83.33±0.88 (65.89)	87.00±1.15 (68.88)	91.33±0.67 (72.87)	87.22±2.31 (69.21)
6H	84.00±0.00 (66.40)	81.67±0.67 (64.63)	84.67±2.60 (67.07)	83.44±0.91 (66.03)	89.33±0.88 (70.94)	84.00±0.58 (66.40)	91.33±1.20 (72.92)	88.22±2.19 (70.09)	90.67±0.33 (72.19)	90.00±0.00 (71.54)	91.33±1.20 (72.92)	90.67±0.38 (72.22)
7H	85.33±0.33 (67.46)	85.00±0.58 (67.19)	89.00±0.58 (70.61)	86.44±1.28 (68.42)	91.33±0.67 (72.87)	87.33±0.33 (69.25)	96.33±0.33 (78.95)	91.66.33±1.86 (72.94)	91.33±0.67 (72.87)	92.67±0.33 (73.87)	96.67±0.33 (79.47)	93.56±4.08 (74.87)
1NH	37.33±0.67 (37.65)	27.33±0.88 (31.50)	35.00±0.58 (36.26)	33.22±3.02 (35.13)	44.00±1.15 (41.54)	38.33±0.67 (38.24)	41.00±0.58 (39.80)	41.11±1.64 (39.86)	45.67±1.20 (42.50)	40.67±0.88 (39.60)	44.33±0.33 (41.73)	43.56±1.49 (41.28)
2NH	43.67±1.45 (41.34)	42.00±1.15 (40.38)	36.67±0.88 (37.25)	40.78±2.11 (39.66)	47.67±1.15 (43.64)	44.67±1.76 (41.92)	40.00±0.58 (39.21)	44.11±2.23 (41.59)	51.67±1.45 (45.94)	42.67±0.67 (40.77)	46.33±0.67 (42.88)	46.89±2.61 (43.19)
3NH	48.33±1.76 (44.03)	47.00±1.00 (43.26)	39.33±0.33 (38.83)	44.89±2.81 (42.04)	50.67±1.45 (45.36)	51.00±1.00 (45.55)	40.00±0.58 (39.21)	47.22±3.61 (43.38)	55.33±0.88 (48.04)	54.00±1.00 (47.28)	48.00±0.58 (43.84)	52.44±2.26 (46.39)
Mean	73.23 (60.38)	72.23 (59.64)	72.83 (60.37)	-	76.60 (62.93)	75.87 (62.34)	76.53 (63.59)	-	78.43 (64.27)	76.97 (63.34)	79.40 (65.73)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

	<u>20h</u>	<u>22h</u>	<u>24h</u>
Colony	(1.24)	(1.11)	(1.11)
Brood Cycle	(0.68)	(0.61)	(0.61)
Colony x Brood Cycle	(2.14)	(1.92)	(1.92)

Col. No.	26 h				28 h				30 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	95.67±0.33 (77.97)	96.33±0.88 (79.11)	96.33±0.88 (79.11)	96.11±0.22 (78.64)	96.33±0.88 (78.95)	96.33±0.88 (79.01)	96.33±0.88 (78.95)	96.33±0.00 (78.97)	96.33±0.88 (78.95)	96.33±0.88 (79.01)	96.33±0.88 (78.95)	96.33±0.00 (78.97)
2H	95.33±0.33 (77.51)	94.67±0.67 (76.67)	97.33±0.33 (80.61)	95.78±0.80 (78.26)	95.33±0.33 (77.51)	95.33±0.67 (77.51)	97.33±0.33 (80.61)	96.00±0.67 (78.56)	95.33±0.33 (77.51)	95.33±0.67 (77.51)	97.33±0.33 (80.61)	96.00±0.67 (78.56)
3H	95.00±0.58 (77.09)	94.33±0.33 (76.21)	96.00±0.58 (78.49)	95.11±0.49 (77.26)	95.00±0.58 (77.09)	95.67±0.33 (77.97)	97.00±0.58 (80.09)	95.89±0.59 (78.38) ^a	95.00±0.58 (77.09)	95.67±0.33 (77.97)	97.00±0.58 (80.09)	95.78±0.49 (78.23)
4H	85.67±1.20 (67.76)	90.33±1.33 (71.94)	93.33±0.33 (75.16)	89.78±2.23 (71.57)	85.67±1.20 (67.76)	90.33±1.33 (71.94)	93.33±0.33 (75.02)	89.78±2.23 (71.57)	85.67±1.20 (67.76)	90.33±1.33 (71.94)	93.33±0.33 (75.02)	89.78±2.23 (71.57)
5H	84.67±0.33 (66.92)	89.33±1.20 (70.96)	92.33±0.33 (73.90)	88.78±2.23 (70.60)	89.00±0.58 (70.61)	89.33±1.20 (70.96)	92.33±0.33 (73.90)	90.22±1.06 (71.83)	90.00±0.00 (71.54)	89.33±1.20 (70.96)	92.33±0.33 (73.90)	90.56±0.91 (72.13)
6H	90.67±0.33 (72.19)	92.33±0.33 (73.90)	95.00±0.00 (77.05)	92.67±1.26 (74.38)	90.67±0.33 (72.19)	93.33±0.88 (75.07)	95.00±0.00 (77.05)	93.00±1.26 (74.77)	90.67±0.33 (72.19)	93.33±0.88 (75.07)	95.00±0.00 (77.05)	93.00±1.26 (74.77)
7H	91.33±0.67 (72.87)	85.00±0.00 (67.19)	96.67±0.33 (79.47)	91.00±3.37 (73.18)	91.33±0.67 (72.87)	88.00±1.00 (69.74)	96.67±0.33 (79.47)	92.00±2.53 (74.03)	91.33±0.67 (72.87)	88.00±1.00 (69.74)	96.67±0.33 (79.47)	92.00±2.53 (74.03)
1NH	56.00±1.15 (48.43)	42.33±0.88 (40.57)	54.00±0.58 (47.28)	50.78±4.26 (45.43)	70.33±0.88 (56.98)	61.67±0.33 (51.73)	70.67±0.67 (51.19)	67.56±3.13 (55.30)	70.33±0.88 (56.98)	61.67±0.33 (51.73)	80.33±0.33 (6.65)	70.78±5.39 (57.45)
2NH	56.67±0.88 (48.81)	51.67±0.33 (45.94)	51.00±1.00 (45.55)	53.11±1.79 (46.77)	70.33±0.88 (56.98)	61.00±1.53 (51.34)	69.33±0.67 (56.35)	66.89±2.96 (54.89)	77.67±1.20 (61.79)	71.33±0.67 (57.61)	76.00±0.58 (60.64)	75.00±1.90 (60.01)
3NH	60.00±1.15 (50.75)	54.00±1.00 (47.28)	54.00±0.58 (47.28)	56.00±2.00 (48.43)	72.00±1.15 (58.04)	74.67±0.33 (59.76)	69.67±0.33 (56.56)	72.11±1.44 (58.12)	76.00±1.00 (60.65)	69.67±2.33 (56.56)	79.00±0.58 (62.70)	74.78±2.86 (59.91)
Mean	81.10 (66.03)	79.03 (64.97)	82.60 (68.36)	-	85.60 (68.90)	84.57 (68.51)	87.77 (71.52)	-	86.83 (69.73)	85.07 (68.80)	90.30 (73.16)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:	<u>26h</u>	<u>28h</u>	<u>30h</u>
Colony	(1.03)	(1.13)	(1.22)
Brood Cycle	(0.56)	(0.62)	(0.67)
Colony x Brood Cycle	(1.78)	(1.96)	(2.12)

The mean brood cells emptied was 91.40 and 47.63 per cent in the hygienic and non-hygienic colonies, respectively. At 26, 28 and 30 h of the pricking in the hygienic colonies, mean brood cells removal was in the range of 92.75, 93.32 and 93.35 per cent and the corresponding values in the non-hygienic colonies were 53.30, 68.96 and 73.52 per cent, respectively.

On the next day, 44 h after the pricking (Table 27, Fig. 21 and 22), after pricking, the non-hygienic colonies in each of the three test brood cycles were continuing to express their hygienic response and the cell emptiness was in the range of 82.00-91.33, 61.67-74.00 and 82.00-89.33 per cent, respectively, with the mean dead brood cells emptied being 80.6 per cent (range: 75.22-83.56%). In the hygienic colonies, the mean dead brood cells emptied was 93.7 per cent.

In the hygienic colonies, the mean dead brood cells emptied after 46 and 48 h was 94.36 and 94.47 per cent, respectively. At 50 h onwards no additional hygienic response was observed in the hygienic colonies. While in the non-hygienic colonies, the mean emptiness was 83.54 and 86.93 per cent, respectively, and it kept on increasing till 54 h after treatment, where a mean of 88.55 per cent was recorded. The data was recorded till 72 h after treatment but no increase was observed in the dead brood cells being emptied (Table 28, and Fig. 21 and 22).

4.6.2 Time interval to attain hygienic behaviour (>80 per cent)

After 20 h of the pricking, in all the 7 hygienic colonies except for the colony 5H, dead brood removal was in the range of 80.89-93.22 per cent. The hygienic colonies on an average took 20.60 h to exhibit the hygienic behaviour (>80% pricked brood removal) whereas, the non-hygienic colonies took 44.60 h to reach > 80%. Thus from this study it is inferred that, after giving the treatment for assessing the hygienic trait of colony, the data is to be recorded 24 h after treatment thus avoiding disturbing the hives normal activities.

4.6.3 Dead brood only uncapped after various time intervals of pricking in three alternate brood cycles

It was observed that within 2 h of the pricking of brood cells, the mean dead brood only uncapped in the hygienic colonies was 14.11 per cent (range: 11.44-21.22%) and 8.40 per cent in the non-hygienic colonies (range: 8.22-8.56%). After 4 hours, brood cells only uncapped in the hygienic colonies ranged between 10.00 to 18.22 per cent and in the non-hygienic colonies 7.89 to 12.67 per cent. After 6 h, in hygienic colonies a wide range 1.67-27.22 per cent of brood cells only uncapped was observed and 7.00 to 12.22 in non-hygienic colonies (Table 29, Fig 23 and 24).

Table 27. Percentage of brood cells emptied by various time intervals after pricking in three alternate brood cycles* starting at 44 h of treatment

Col. No.	44 h				46 h				48 h			
	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean
1H	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)
2H	95.33±0.33 (77.51)	95.33±0.67 (77.51)	97.33±0.33 (80.61)	96.00±0.67 (78.54)	95.33±0.33 (77.51)	95.33±0.67 (77.51)	97.33±0.33 (80.61)	96.00±0.67 (78.54)	95.33±0.33 (77.51)	95.33±0.67 (77.51)	97.33±0.33 (80.61)	96.00±0.67 (78.54)
3H	95.00±0.58 (77.09)	95.67±0.33 (77.97)	97.00±0.00 (79.99)	95.89±0.59 (78.35)	95.00±0.58 (77.09)	95.67±0.33 (77.97)	97.00±0.00 (79.99)	95.89±0.59 (78.35)	95.00±0.58 (77.09)	95.67±0.33 (77.97)	97.00±0.00 (79.99)	95.89±0.59 (78.35)
4H	88.00±1.73 (69.80)	90.33±1.33 (71.94)	93.33±0.33 (75.02)	90.56±1.53 (72.25)	90.67±1.45 (72.28)	92.67±0.33 (74.27)	93.33±0.33 (75.02)	92.22±0.80 (73.85)	90.67±1.45 (72.28)	93.00±0.58 (74.65)	93.33±0.33 (75.16)	92.33±0.83 (73.98)
5H	90.00±0.00 (71.54)	89.33±1.20 (70.96)	92.33±0.33 (73.90)	90.56±0.91 (72.13)	90.00±0.00 (71.54)	91.67±0.67 (73.22)	92.33±0.33 (73.90)	91.33±0.69 (72.89)	91.33±0.67 (72.87)	91.67±0.67 (73.22)	92.33±0.33 (73.90)	91.78±0.28 (73.33)
6H	91.67±1.20 (73.28)	93.33±0.88 (75.07)	95.00±0.00 (77.05)	93.33±0.95 (75.13)	92.00±1.00 (73.61)	94.33±0.67 (76.24)	95.00±0.00 (77.05)	93.78±0.91 (75.63)	92.67±0.88 (74.31)	94.33±0.67 (76.24)	95.00±0.00 (77.05)	94.00±0.68 (75.87)
7H	92.67±1.76 (74.50)	88.00±1.00 (69.74)	96.67±0.33 (79.47)	92.44±2.51 (74.57)	94.33±1.20 (76.34)	92.00±1.00 (73.61)	96.67±0.33 (79.47)	94.33±1.36 (76.47)	94.33±1.20 (76.34)	92.00±1.00 (73.61)	96.67±0.33 (79.47)	94.33±1.36 (76.47)
1NH	82.00±1.00 (64.89)	61.67±0.33 (51.73)	82.00±1.53 (64.91)	75.22±6.77 (60.51)	82.00±1.00 (64.89)	76.33±0.33 (60.87)	85.00±0.00 (67.19)	81.11±2.55 (64.31)	90.67±0.33 (72.19)	79.67±0.88 (63.18)	87.33±1.20 (69.16)	85.89±3.25 (68.18)
2NH	91.33±0.88 (72.89)	71.33±0.67 (57.61)	88.00±0.58 (69.71)	83.56±6.19 (66.74)	91.33±0.88 (72.89)	77.67±0.88 (61.78)	88.00±0.58 (69.71)	85.67±4.10 (68.13)	92.67±0.33 (74.27)	81.33±1.20 (64.40)	88.00±0.58 (69.71)	87.33±3.91 (69.46)
3NH	86.00±1.00 (68.03)	74.00±0.00 (59.32)	89.33±0.67 (70.93)	83.11±4.65 (66.09)	86.00±1.00 (68.03)	76.33±0.33 (60.87)	89.33±0.67 (70.93)	83.89±3.90 (66.61)	88.67±0.88 (70.33)	84.67±0.33 (66.92)	89.33±0.67 (70.93)	87.56±1.44 (69.39)
Mean	90.90 (72.95)	85.60 (69.18)	92.80 (75.16)	-	91.37 (73.42)	88.90 (71.63)	93.10 (75.39)	-	92.83 (74.72)	90.47 (72.77)	92.73 (75.58)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

	<u>44h</u>	<u>46h</u>	<u>48h</u>
Colony	(1.32)	(1.08)	(1.12)
Brood Cycle	(0.72)	(0.59)	(0.62)
Colony x Brood Cycle	(2.28)	(1.87)	(1.94)

Col. No.	50 h				52 h				54 h			
	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean
1H	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)
2H	95.33±0.33 (77.51)	95.33±0.67 (77.51)	97.33±0.33 (80.61)	96.00±0.67 (78.54)	95.33±0.33 (77.51)	95.33±0.67 (77.51)	97.33±0.33 (80.61)	96.00±0.67 (78.54)	95.33±0.33 (77.51)	95.33±0.67 (77.51)	97.33±0.33 (80.61)	96.00±0.67 (78.54)
3H	95.00±0.58 (77.09)	95.67±0.33 (77.97)	97.00±0.00 (79.99)	95.89±0.59 (78.35)	95.00±0.58 (77.09)	95.67±0.33 (77.97)	97.00±0.00 (79.99)	95.89±0.59 (78.35)	95.00±0.58 (77.09)	95.67±0.33 (77.97)	97.00±0.00 (79.99)	95.89±0.59 (78.35)
4H	90.67±1.45 (72.28)	93.00±0.58 (74.65)	93.33±0.33 (75.02)	92.33±0.83 (73.98)	90.67±1.45 (72.28)	93.00±0.58 (74.65)	93.33±0.33 (75.16)	92.33±0.83 (73.98)	90.67±1.45 (72.28)	93.00±0.58 (74.65)	93.33±0.33 (75.16)	92.33±0.83 (73.98)
5H	91.33±0.67 (72.87)	91.67±0.67 (73.22)	92.33±0.33 (73.90)	91.78±0.28 (73.33)	91.33±0.67 (72.87)	91.67±0.67 (73.22)	92.33±0.33 (73.90)	91.78±0.28 (73.33)	91.33±0.67 (72.87)	91.67±0.67 (73.22)	92.33±0.33 (73.90)	91.78±0.28 (73.33)
6H	92.67±0.88 (74.31)	94.33±0.67 (76.24)	95.00±0.00 (77.05)	94.00±0.68 (75.87)	92.67±0.88 (74.31)	94.33±0.67 (76.24)	95.00±0.00 (77.05)	94.00±0.68 (75.87)	92.67±0.88 (74.31)	94.33±0.67 (76.24)	95.00±0.00 (77.05)	94.00±0.68 (75.87)
7H	94.33±1.20 (76.34)	92.00±1.00 (73.61)	96.67±0.33 (79.47)	94.33±1.36 (76.47)	94.33±1.20 (76.34)	92.00±1.00 (73.61)	96.67±0.33 (79.47)	94.33±1.36 (76.47)	94.33±1.20 (76.34)	92.00±1.00 (73.61)	96.67±0.33 (79.47)	94.33±1.36 (76.47)
1NH	90.67±0.33 (72.19)	80.33±1.20 (63.65)	87.33±1.20 (69.16)	86.11±3.04 (68.34)	90.67±0.33 (72.19)	82.00±0.00 (64.87)	87.33±1.20 (69.16)	86.67±2.52 (68.74)	90.67±0.33 (72.19)	82.00±0.33 (65.12)	87.33±1.20 (69.16)	86.78±2.43 (68.82)
2NH	92.67±0.33 (74.27)	84.00±1.00 (66.42)	88.00±0.58 (69.71)	88.22±2.50 (70.13)	92.67±0.33 (74.27)	87.67±0.33 (69.71)	88.00±0.58 (69.71)	89.44±1.62 (71.15)	92.67±0.33 (74.26)	87.67±1.00 (69.41)	88.00±0.58 (69.71)	89.44±1.62 (71.13)
3NH	92.67±0.67 (74.29)	84.67±0.33 (66.92)	89.33±0.67 (70.93)	88.89±2.31 (70.71)	92.67±0.67 (74.29)	86.33±0.33 (68.28)	89.33±0.67 (70.93)	89.44±1.84 (71.17)	92.67±0.67 (74.26)	86.33±0.33 (68.28)	89.33±0.67 (70.93)	89.44±1.84 (71.16)
Mean	93.23 (75.11)	90.80 (73.01)	93.33 (75.58)	-	93.23 (75.11)	91.50 (73.58)	93.33 (75.58)	-	93.23 (75.11)	91.53 (73.60)	93.33 (75.58)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

	<u>50h</u>	<u>52h</u>	<u>54h</u>
Colony	(1.05)	(1.05)	(1.06)
Brood Cycle	(0.58)	(0.58)	(0.58)
Colony x Brood Cycle	(1.83)	(1.83)	(1.83)

Table 28. Percentage of brood cells emptied by various time intervals after pricking in three alternate brood cycles* starting at 68 h of treatment

Col. No.	68 h				70 h				72 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)
2H	95.33±0.33 (77.51)	95.33±0.67 (77.51)	97.33±0.33 (80.61)	96.00±0.67 (78.54)	95.33±0.33 (77.51)	95.33±0.67 (77.51)	97.33±0.33 (80.61)	96.00±0.67 (78.54)	95.33±0.33 (77.51)	95.33±0.67 (77.51)	97.33±0.33 (80.61)	96.00±0.67 (78.54)
3H	95.00±0.58 (77.09)	95.67±0.33 (77.97)	97.00±0.00 (79.99)	95.89±0.59 (78.35)	95.00±0.58 (77.09)	95.67±0.33 (77.97)	97.00±0.00 (79.99)	95.89±0.59 (78.35)	95.00±0.58 (77.09)	95.67±0.33 (77.97)	97.00±0.00 (79.99)	95.89±0.59 (78.35)
4H	90.67±1.45 (72.28)	93.00±0.58 (74.65)	93.33±0.33 (75.16)	92.33±0.83 (73.98)	90.67±1.45 (72.28)	93.00±0.58 (74.65)	93.33±0.33 (75.16)	92.33±0.83 (73.98)	90.67±1.45 (72.28)	93.00±0.58 (74.65)	93.33±0.33 (75.16)	92.33±0.83 (73.98)
5H	91.33±0.67 (72.87)	91.67±0.67 (73.22)	92.33±0.33 (73.90)	91.78±0.28 (73.33)	91.33±0.67 (72.87)	91.67±0.67 (73.22)	92.33±0.33 (73.90)	91.78±0.28 (73.33)	91.33±0.67 (72.87)	91.67±0.67 (73.22)	92.33±0.33 (73.90)	91.78±0.28 (73.33)
6H	92.67±0.88 (74.31)	94.33±0.67 (76.24)	95.00±0.00 (77.05)	94.00±0.68 (75.87)	92.67±0.88 (74.31)	94.33±0.67 (76.24)	95.00±0.00 (77.05)	94.00±0.68 (75.87)	92.67±0.88 (74.31)	94.33±0.67 (76.24)	95.00±0.00 (77.05)	94.00±0.68 (75.87)
7H	94.33±1.20 (76.34)	92.00±1.00 (73.61)	96.67±0.33 (79.47)	94.33±1.36 (76.47)	94.33±1.20 (76.34)	92.00±1.00 (73.61)	96.67±0.33 (79.47)	94.33±1.36 (76.47)	94.33±1.20 (76.34)	92.00±1.00 (73.61)	96.67±0.33 (79.47)	94.33±1.36 (76.47)
1NH	90.67±0.33 (72.19)	82.00±0.33 (65.12)	87.33±1.20 (69.16)	86.78±2.43 (68.82)	90.67±0.33 (72.19)	82.00±0.33 (65.12)	87.33±1.20 (69.16)	86.78±2.43 (68.82)	90.67±0.33 (72.19)	82.00±0.33 (65.12)	87.33±1.20 (69.16)	86.78±2.43 (68.82)
2NH	92.67±0.33 (74.26)	87.67±1.00 (69.41)	88.00±0.58 (69.71)	89.44±1.62 (71.13)	92.67±0.33 (74.26)	87.67±1.00 (69.41)	88.00±0.58 (69.71)	89.44±1.62 (71.13)	92.67±0.33 (74.26)	87.67±1.00 (69.41)	88.00±0.58 (69.71)	89.44±1.62 (71.13)
3NH	92.67±0.67 (74.26)	86.33±0.33 (68.28)	89.33±0.67 (70.93)	89.44±1.84 (71.16)	92.67±0.67 (74.26)	86.33±0.33 (68.28)	89.33±0.67 (70.93)	89.44±1.84 (71.16)	92.67±0.67 (74.26)	86.33±0.33 (68.28)	89.33±0.67 (70.93)	89.44±1.84 (71.16)
Mean	93.23 (75.11)	91.53 (73.60)	93.33 (75.58)	-	93.23 (75.11)	91.53 (73.60)	93.33 (75.58)	-	93.23 (75.11)	91.53 (73.60)	93.33 (75.58)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:	<u>68h</u>	<u>70h</u>	<u>72h</u>
Colony	(1.06)	(1.06)	(1.06)
Brood Cycle	(0.58)	(0.58)	(0.58)
Colony x Brood Cycle	(1.83)	(1.83)	(1.83)

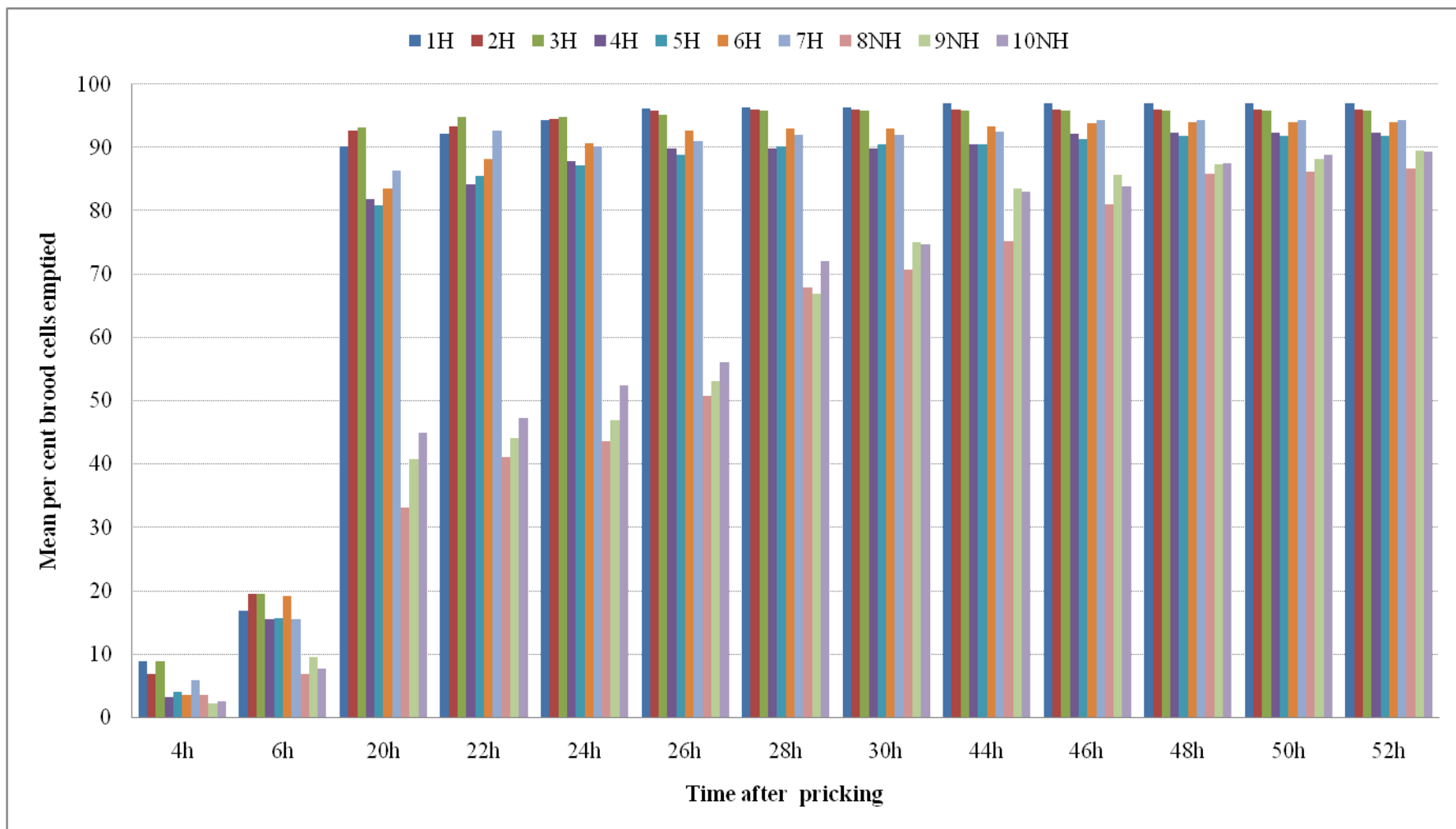


Fig. 21 Mean percentage of brood cells emptied by various time intervals after pricking in three alternate brood cycles

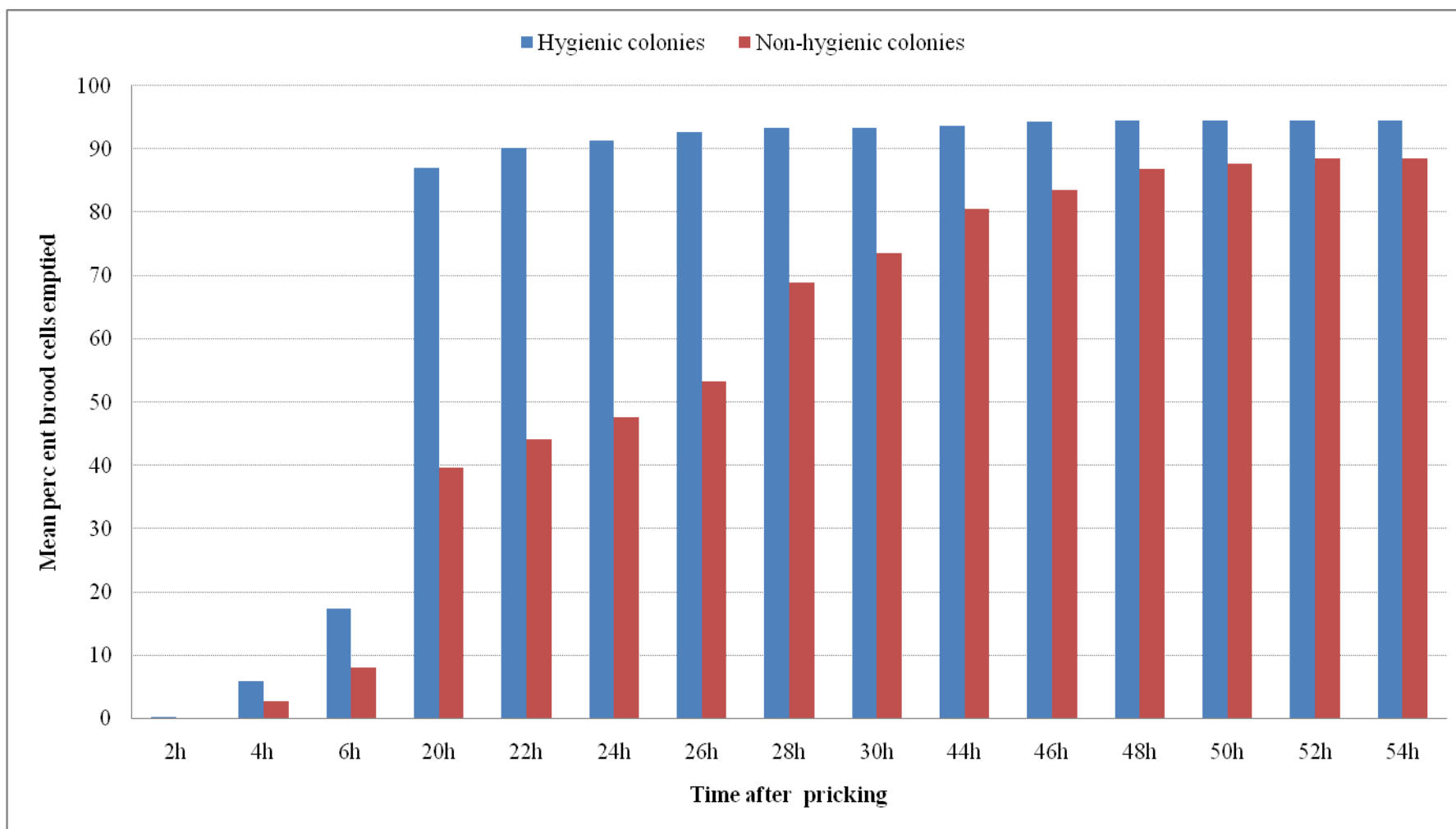


Fig. 22 Mean percentage of brood cells emptied by various time intervals after pricking in three alternate brood cycles in hygienic and non-hygienic colonies

Table 29. Percentage of brood cells only uncapped by various time intervals in three alternate brood cycles*

Col. No.	2 h				4 h				6 h			
	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	13.00±0.58 (21.12)	14.67±0.33 (22.51)	16.33±1.20 (23.80)	14.67±0.95 (22.47)	21.33±0.88 (27.49)	16.33±1.45 (23.79)	17.00±0.58 (24.33)	18.22±1.57 (25.20)	27.00±0.58 (31.29)	27.67±0.33 (31.72)	27.00±1.15 (31.29)	27.22±0.22 (31.43)
2H	19.67±1.45 (26.29)	23.00±0.58 (28.64)	21.00±0.58 (27.26)	21.22±0.96 (27.40)	13.67±0.67 (21.68)	26.67±0.88 (31.07)	19.33±0.88 (26.06)	19.89±3.76 (26.27)	8.67±0.67 (17.09)	20.00±1.53 (26.52)	20.00±0.58 (26.52)	16.22±3.78 (23.39)
3H	11.00±0.58 (19.35)	16.33±0.67 (23.82)	15.00±0.58 (22.77)	14.11±1.59 (21.98)	8.33±0.88 (16.73)	10.67±0.67 (19.04)	11.00±0.00 (19.36)	10.00±0.84 (18.38)	3.67±0.33 (11.01)	0.67±0.67 (2.71)	1.33±0.88 (5.23)	1.89±0.91 (6.32)
4H	9.67±0.88 (18.07)	13.67±0.88 (21.67)	14.00±0.00 (21.96)	12.44±1.39 (20.57)	13.33±0.88 (21.39)	16.00±0.58 (23.56)	14.00±0.58 (21.96)	14.44±0.80 (22.30)	6.33±0.33 (14.56)	6.67±0.88 (14.89)	6.33±0.88 (14.50)	6.44±0.11 (14.65)
5H	13.67±0.67 (21.68)	11.00±0.58 (19.35)	13.00±0.58 (21.12)	12.55±0.81 (20.71)	10.67±0.67 (19.04)	10.67±0.67 (19.04)	11.00±1.15 (19.31)	10.78±0.11 (19.13)	1.67±0.88 (6.03)	2.67±0.33 (9.36)	5.33±0.88 (13.26)	3.22±1.09 (9.55)
6H	12.33±0.88 (20.53)	10.00±0.58 (18.41)	12.00±0.58 (20.25)	11.44±0.72 (19.73)	13.00±0.58 (21.12)	12.67±0.88 (20.82)	14.33±0.33 (22.23)	13.33±0.51 (21.39)	1.67±0.88 (6.03)	1.67±0.88 (6.03)	1.67±0.33 (7.33)	1.67±0.00 (6.46)
7H	11.67±0.88 (19.94)	11.33±0.67 (19.65)	14.00±0.58 (21.96)	12.33±0.84 (20.51)	9.00±0.58 (17.43)	9.33±0.67 (17.76)	12.67±0.88 (20.82)	10.33±1.17 (18.67)	5.00±0.58 (12.87)	1.33±0.67 (5.42)	8.00±0.58 (16.40)	4.78±1.93 (11.56)
1NH	9.33±0.88 (17.75)	5.67±0.67 (13.72)	10.33±0.88 (18.71)	8.44±1.40 (16.73)	9.67±1.20 (18.04)	8.00±0.58 (16.40)	6.00±0.58 (14.14)	7.89±1.06 (16.20)	9.33±0.67 (17.76)	7.67±0.67 (16.04)	16.33±1.33 (23.80)	11.11±2.65 (19.20)
2NH	12.00±1.00 (20.23)	3.33±1.20 (10.06)	9.33±0.67 (17.76)	8.22±2.57 (16.02)	13.33±0.88 (21.39)	7.00±0.58 (15.32)	11.67±0.88 (19.94)	10.67±1.90 (18.88)	9.33±0.33 (17.78)	3.67±0.88 (10.86)	8.00±0.00 (16.42)	7.00±1.71 (15.02)
3NH	10.00±0.58 (18.41)	11.00±0.58 (19.35)	4.67±1.45 (12.13)	8.56±1.95 (16.63)	9.33±0.67 (17.75)	10.33±0.33 (18.74)	18.33±0.33 (25.34)	12.67±2.85 (20.61)	8.67±0.67 (17.09)	6.00±0.58 (14.14)	22.00±1.15 (27.95)	12.22±4.95 (19.73)
Mean	12.23 (20.34)	12.00 (19.72)	12.97 (20.77)	-	12.17 (20.21)	12.77 (20.55)	13.53 (21.35)	-	8.13 (15.15)	7.80 (13.77)	11.60 (18.27)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

	<u>2h</u>	<u>4h</u>	<u>6h</u>
Colony	(1.37)	(1.10)	(2.41)
Brood Cycle	(0.75)	(0.60)	(1.32)
Colony x Brood Cycle	(2.31)	(1.90)	(4.17)

On next day, i.e. after 20 h of the pricking, the mean percentage of brood cells only uncapped in the hygienic colonies reduced to 7.23 per cent (range: 1.56 to 12.56%). In non-hygienic colonies (1NH, 2NH and 3NH) as the adult worker honey bees were slow in detecting the cues from the pricked brood cells, mean brood cells only uncapped was 40.13 per cent (range: 36.11-46.89%). After 24 h, in the hygienic colonies, the mean brood only uncapped was 4.62 per cent (range: 1.44 to 8.33%), and the corresponding value in non-hygienic colonies was 39.22 per cent (range: 36.11-43.67%). After 26, 28 and 30 h, in the hygienic colonies the mean brood cells only uncapped were 4.21, 3.91 and 3.86 per cent and the corresponding values in the non-hygienic colonies were 36.48, 23.19 and 18.67 per cent, respectively (Table 30, Fig. 23 and 24).

After 44 h, the mean brood cells only uncapped was 4.83 and 12.67 per cent in hygienic and non-hygienic colonies, respectively. After 46, 48, 50 and 52 h (Table 31, Fig. 23 and 24) in the hygienic colonies the mean brood cells only uncapped were 3.40, 3.35, 3.24 and 3.22 per cent and in the non-hygienic colonies, it was 9.96, 7.30, 6.63 and 5.85 per cent, respectively. The data was recorded up to 72 h after treatment, but no additional change was observed in the performance of uncapping of pricked brood (Table 32, Fig 23 and 24).

4.6.4 Dead brood remaining capped after various time intervals of pricking in three alternate brood cycles

The mean brood cells remaining capped after 2, 4 and 6 h, were 85.69, 80.27 and 73.87 per cent in the hygienic colonies and 91.59, 86.85 and 81.74 per cent in the non-hygienic colonies. (Table 33, Fig 25 and 26).

On the next day i.e. after 20 h, the mean brood cells remaining capped in the hygienic colonies was 6.06 per cent (range: 2.56-8.89%) and in the non-hygienic colonies 20.22 per cent (range: 19-21.78%). At this time period, the hygienic colonies have expressed hygienic traits >80.00%, thus the low per cent of capped brood cells observed. After 22, 24 and 26 h in the hygienic colonies the mean brood cells remaining capped were 4.35, 3.55 and 2.98 per cent (Table 34, Fig 25 and 26). After 22, 24, 26, 28 and 30 h in the non-hygienic colonies, the mean brood cells remaining capped were 17.29, 13.11, 9.67, 7.96 and 7.22 per cent.

After 44, 46 and 48 h (Table 35, Fig 25 and 26) in the hygienic colonies, the mean brood cells remaining capped were 2.46, 2.27 and 1.89 per cent. After 44, 46, 48 and 50 h of the pricking, in the non-hygienic colonies, the mean dead brood cells remaining capped was 6.74, 6.44, 5.78 and 5.63 per cent. After 72 h of the pricking (Table 36, Fig 25 and 26) no change in the hygienic response was observed.

The present findings are in line with that of Pereira *et al* (2013) who reported that after one hour of the pricking, hygienic bees had removed 43 per cent, whereas, the non-hygienic bees removed only 25 per cent. Hygienic bees completed the removal in 15 h, whereas the non-hygienic bees took over 30 h. Rasolofoarivao *et al* 2015 tested a total of 403 colonies from three regions and observed variability in hygienic responses. In the first 4 h, colonies detected a mean of 50 per cent of the pricked brood. After 4 h, all the colonies had uncapped the brood. After 6 h, colonies displayed a wide range of detected cells (32- 100%), uncapped cells (0-100%) and cleaned cells (0- 82%).

Table 30. Percentage of brood cells only uncapped by various time intervals in three alternate brood cycles* starting at 20 h of treatment

Col. No.	20 h				22 h				24 h			
	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle 1	Brood Cycle II	Brood Cycle III	Mean
1H	2.33±0.33 (8.74)	1.33±0.33 (6.53)	1.00±0.58 (4.62)	1.56±0.67 (6.63)	2.33±0.33 (8.74)	1.33±0.33 (6.53)	1.00±0.58 (4.62)	1.56±0.67 (6.63)	2.33±0.33 (8.74)	1.33±0.33 (6.53)	1.00±0.58 (4.62)	1.56±0.67 (6.63)
2H	5.67±1.76 (13.45)	5.00±0.00 (12.92)	3.00±0.00 (9.97)	4.56±1.46 (12.11)	5.33±0.88 (13.26)	5.00±0.00 (12.92)	3.00±0.00 (9.97)	4.44±1.39 (12.05)	4.67±0.88 (12.35)	4.00±0.33 (11.53)	3.00±0.00 (9.97)	3.89±1.13 (11.32)
3H	4.33±0.33 (11.99)	6.00±0.58 (14.14)	2.33±0.33 (8.74)	4.22±0.87 (11.63)	4.33±0.33 (11.99)	4.33±0.33 (11.99)	1.33±0.67 (6.53)	3.33±0.44 (10.15)	4.33±0.33 (11.99)	4.33±0.33 (11.99)	1.33±0.67 (6.53)	3.33±0.44 (10.15)
4H	14.00±0.58 (21.96)	13.33±0.67 (21.40)	8.67±1.33 (17.01)	12.00±3.79 (20.12)	16.67±0.33 (24.08)	9.33±0.67 (17.76)	6.67±0.88 (14.89)	10.89±4.43 (18.91)	15.33±0.88 (23.02)	6.33±0.67 (14.53)	3.33±1.67 (8.61)	8.33±4.10 (15.39)
5H	12.67±1.45 (20.78)	7.00±0.58 (15.31)	11.00±0.58 (19.35)	10.22±1.69 (18.48)	7.00±0.58 (15.31)	5.67±0.33 (13.75)	6.00±1.00 (14.08)	6.22±0.40 (14.38)	7.33±0.33 (15.70)	5.67±0.33 (13.75)	4.00±0.00 (11.53)	5.67±1.16 (13.66)
6H	11.67±1.20 (19.92)	12.33±0.67 (20.54)	13.67±0.67 (21.68)	12.56±0.38 (20.72)	10.67±0.88 (19.02)	13.33±0.88 (21.39)	2.00±0.00 (8.13)	8.67±2.14 ^f (16.18)	9.33±0.33 (17.78)	8.00±0.00 (16.42)	2.00±0.00 (8.13)	6.44±1.60 (14.11)
7H	7.67±0.33 (16.06)	8.67±0.33 (17.11)	7.67±0.67 (16.04)	8.00±1.84 (16.40)	4.67±0.33 (12.45)	6.00±1.00 (14.08)	6.00±1.00 (14.08)	5.56±0.44 (13.60)	4.67±0.33 (12.45)	4.67±0.33 (12.45)	1.00±0.58 (4.62)	3.44±0.89 (9.84)
1NH	43.33±0.88 (41.15)	56.33±0.88 (48.62)	41.00±1.00 (39.80)	46.89±4.76 (43.19)	39.67±0.33 (39.02)	47.33±0.67 (43.45)	38.00±0.00 (38.04)	41.67±2.87 (40.17)	43.67±0.33 (41.34)	46.00±0.58 (42.69)	41.33±0.67 (39.99)	43.67±1.35 (41.34)
2NH	37.00±1.00 (37.45)	34.00±1.15 (35.65)	41.33±0.33 (39.99)	37.44±2.12 (37.70)	34.67±0.67 (36.05)	34.33±2.19 (35.83)	43.00±1.15 (40.96)	37.33±2.84 (37.62)	35.00±0.58 (36.26)	36.00±0.00 (36.86)	42.67±0.88 (40.77)	37.89±2.41 (37.96)
3NH	33.33±0.88 (35.25)	30.67±0.33 (33.61)	44.33±0.33 (41.73)	36.11±4.17 (36.86)	33.33±1.33 (35.24)	27.33±0.33 (31.51)	49.33±0.67 (44.60)	36.67±6.57 (37.12)	35.00±1.33 (36.25)	30.67±0.67 (33.61)	42.67±0.67 (40.77)	36.11±3.51 (36.87)
Mean	17.20 22.68	17.47 22.58	17.40 21.89	-	15.87 (21.52)	15.40 (20.93)	15.63 (19.60)	-	16.17 (21.60)	14.70 (20.03)	14.23 (17.55)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:	<u>20h</u>	<u>22h</u>	<u>24h</u>
Colony	(1.37)	(1.27)	(1.81)
Brood Cycle	NS	(0.70)	(0.99)
Colony x Brood Cycle	(2.37)	(2.21)	(3.14)

Col. No.	26 h				28 h				30 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	2.67±0.67 (9.26)	0.67±0.67 (2.71)	0.00±0.00 (0.00)	1.11±0.80 (3.99)	2.00±0.00 (8.132)	0.67±0.67 (2.71)	0.00±0.00 (0.00)	0.89±0.59 (3.61)	2.00±0.00 (8.132)	0.67±0.67 (2.71)	0.00±0.00 (0.00)	0.89±0.59 (3.61)
2H	4.33±0.33 (11.99)	4.33±0.33 (11.99)	1.33±0.33 (6.53)	3.33±1.00 (10.17)	4.33±1.33 (11.99)	3.67±0.33 (11.01)	1.33±0.33 (6.53)	3.11±0.91 (9.85)	4.33±1.33 (11.99)	3.67±0.33 (11.01)	1.33±0.33 (6.53)	3.11±0.91 (9.85)
3H	4.33±0.33 (11.99)	3.67±0.33 (10.85)	1.00±0.58 (4.62)	3.00±1.02 (9.16)	4.33±0.33 (11.99)	3.00±0.58 (9.88)	1.00±0.58 (4.62)	2.78±0.97 (8.83)	4.33±0.33 (11.99)	3.00±0.33 (9.88)	1.00±0.58 (4.62)	2.78±0.97 (8.83)
4H	11.67±0.88 (19.94)	5.67±0.33 (13.75)	2.00±1.00 (6.65)	6.44±2.82 (13.45)	11.67±0.88 (19.94)	5.67±0.33 (13.75)	2.00±1.00 (6.65)	6.44±2.82 (13.45)	11.67±0.88 (19.94)	5.67±0.33 (13.75)	2.00±1.00 (6.65)	6.44±2.82 (13.45)
5H	6.33±0.67 (14.53)	4.67±0.33 (12.45)	3.67±0.33 (11.01)	4.89±0.78 (12.67)	6.00±0.58 (14.14)	4.67±0.33 (12.45)	3.67±0.33 (11.01)	4.78±0.67 (12.54)	5.00±0.58 (12.87)	4.67±0.33 (12.45)	3.67±0.33 (11.01)	4.44±0.40 (12.11)
6H	9.33±0.33 (17.78)	6.33±0.88 (14.50)	0.00±0.00 (0.00)	5.22±2.75 (10.76)	9.33±0.33 (17.78)	5.33±0.33 (13.33)	0.00±0.00 (0.00)	4.89±2.70 (10.37)	9.33±0.33 (17.78)	5.33±0.33 (13.33)	0.00±0.00 (0.00)	4.89±2.70 (10.37)
7H	4.67±0.33 (12.45)	10.67±0.67 (19.04)	1.00±0.58 (4.62)	5.44±2.82 (12.04)	4.67±0.33 (12.45)	7.67±0.33 (16.07)	1.00±0.58 (4.62)	4.44±1.93 (11.05)	4.67±0.33 (12.45)	7.67±0.33 (16.06)	1.00±0.58 (4.62)	4.44±1.93 (11.05)
1NH	33.33±1.86 (35.24)	44.67±0.88 (41.92)	39.00±1.00 (38.63)	39.00±3.27 (38.59)	23.00±1.53 (28.62)	29.00±0.58 (32.57)	22.33±1.20 (28.17)	24.78±2.12 (29.79)	23.00±1.53 (28.62)	29.00±0.58 (32.57)	13.67±0.88 (21.67)	21.89±4.46 (27.62)
2NH	29.00±1.73 (32.55)	33.33±1.86 (35.24)	41.67±0.88 (40.19)	34.67±3.72 (35.99)	20.67±1.20 (27.01)	28.33±0.67 (32.14)	23.33±0.88 (28.87)	24.11±2.25 (29.34)	16.67±0.67 (24.08)	19.67±0.67 (26.31)	17.33±0.88 (24.58)	17.89±0.91 (24.99)
3NH	29.67±0.88 (32.98)	38.00±0.00 (38.04)	39.67±0.67 (39.02)	35.78±3.09 (36.68)	20.00±1.00 (26.54)	17.33±0.67 (24.59)	17.33±0.33 (29.77)	20.67±2.34 (26.96)	16.00±0.58 (23.56)	17.33±0.67 (24.59)	15.33±0.33 (23.04)	16.22±0.59 (23.73)
Mean	13.53 (19.87)	15.20 (20.05)	12.93 (15.13)	-	10.60 (17.86)	10.53 (16.85)	7.93 (12.02)	-	9.70 (17.14)	9.67 (16.27)	5.53 (10.27)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:	<u>26h</u>	<u>28h</u>	<u>30h</u>
Colony	(1.94)	(1.85)	(1.84)
Brood Cycle	(1.06)	(1.01)	(1.01)
Colony x Brood Cycle	(3.35)	(3.21)	(3.18)

Table 31. Percentage of brood cells only uncapped by various time intervals in three alternate brood cycles* starting at 44 h of treatment

Col. No.	44 h				46 h				48 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	1.67±1.67 (4.31)	0.33±0.33 (1.91)	3.00±1.53 (8.15)	1.67±0.78 (4.79)	1.67±1.67 (4.31)	2.00±1.53 (6.22)	3.00±1.53 (8.15)	2.22±0.62 (6.22)	1.67±1.67 (4.31)	2.00±1.53 (6.22)	3.00±1.53 (8.15)	2.22±0.62 (6.22)
2H	4.67±0.88 (12.35)	3.67±1.67 (10.53)	1.33±0.33 (6.53)	3.22±6.97 (9.81)	4.67±0.88 (12.35)	4.33±1.45 (11.66)	1.33±0.33 (6.53)	3.44±0.51 (10.18)	4.33±0.67 (11.93)	3.67±1.67 (10.53)	1.33±0.33 (6.53)	3.11±0.38 (9.67)
3H	4.67±0.88 (12.35)	3.00±0.00 (9.97)	2.00±1.15 (6.55)	3.22±0.79 (9.62)	4.67±0.33 (9.97)	3.00±1.53 (8.15)	2.00±1.15 (6.55)	3.22±0.96 (9.62)	4.67±0.33 (12.35)	3.00±1.53 (9.97)	2.00±1.53 (6.55)	3.22±0.96 (9.62)
4H	9.33±1.76 (17.62)	5.67±0.33 (13.75)	2.00±1.00 (6.65)	5.67±2.11 (12.67)	7.33±0.88 (15.65)	4.00±0.58 (11.47)	2.00±1.00 (6.65)	4.44±1.56 (11.26)	7.33±0.88 (15.65)	3.67±0.33 (11.01)	2.00±1.00 (6.55)	4.33±1.57 (11.10)
5H	5.00±0.58 (12.87)	4.67±0.33 (12.45)	3.67±0.33 (11.01)	4.44±0.39 (12.11)	5.00±0.58 (12.87)	2.33±0.67 (8.55)	3.67±0.33 (11.01)	3.67±0.77 (11.81)	5.00±0.58 (12.87)	2.33±0.67 (8.56)	3.67±0.33 (11.10)	3.67±0.95 (10.81)
6H	8.33±1.20 (16.68)	5.33±0.33 (13.33)	0.00±0.00 (0.00)	4.56±2.43 (10.00)	8.00±1.00 (16.36)	5.67±0.67 (13.72)	0.00±0.00 (0.00)	4.56±2.38 (10.03)	7.33±0.88 (15.65)	5.67±0.67 (13.72)	0.00±0.00 (0.00)	4.33±1.06 (9.79)
7H	4.33±0.33 (11.99)	7.67±0.33 (16.06)	1.00±0.58 (4.62)	4.33±1.93 (10.89)	4.00±0.58 (11.47)	3.67±0.33 (11.01)	1.00±0.58 (4.62)	2.89±0.95 (9.04)	4.00±0.58 (11.47)	3.67±0.33 (11.01)	1.00±0.58 (4.62)	2.89±1.28 (9.04)
1NH	13.33±0.88 (21.39)	29.00±0.58 (32.57)	12.00±2.52 (20.03)	18.11±5.46 (24.66)	13.33±0.88 (21.39)	15.00±0.58 (22.77)	9.00±1.00 (17.40)	12.44±1.79 (20.52)	5.33±0.88 (13.26)	12.67±1.20 (20.80)	6.67±0.33 (14.95)	8.22±2.26 (16.34)
2NH	4.00±0.58 (11.47)	19.67±0.67 (26.31)	5.33±0.33 (13.33)	9.67±5.03 (17.04)	4.00±0.58 (11.47)	15.00±1.15 (22.75)	5.33±0.33 (13.33)	8.11±3.47 (15.85)	3.00±0.00 (9.97)	12.00±1.53 (20.18)	5.33±0.33 (13.33)	6.78±2.70 (14.49)
3NH	8.33±0.88 (16.73)	17.33±0.67 (24.59)	5.00±0.00 (12.92)	10.22±3.68 (18.08)	8.33±0.88 (16.73)	14.67±0.33 (22.51)	5.00±0.00 (12.92)	9.33±2.84 (17.38)	9.33±0.33 (17.78)	6.33±0.33 (14.56)	5.00±0.00 (12.92)	6.89±1.28 (15.08)
Mean	6.37 (13.78)	9.63 (16.15)	3.53 (8.98)	1.67±0.78 (4.79)	6.10 (13.49)	6.97 (14.21)	3.23 (8.52)	-	5.20 (12.52)	5.50 (12.66)	3.00 (8.47)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:	<u>44h</u>	<u>46h</u>	<u>48h</u>
Colony	(2.86)	(2.95)	(2.95)
Brood Cycle	(1.57)	(1.61)	(1.62)
Colony x Brood Cycle	(4.96)	(5.10)	(5.11)

Col. No.	50 h				52 h				54 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	1.67±1.67 (4.31)	0.67±0.67 (2.71)	3.00±1.53 (8.15)	1.78±0.49 (5.05)	1.67±1.67 (4.31)	2.00±1.53 (6.22)	3.00±1.53 (8.15)	2.22±0.40 (6.22)	1.67±1.67 (4.31)	2.00±1.53 (6.22)	3.00±1.53 (8.15)	2.22±0.40 (6.22)
2H	4.33±0.67 (11.93)	3.67±1.67 (10.53)	1.33±0.33 (6.53)	3.11±0.38 (9.67)	4.33±0.67 (11.93)	3.67±1.67 (10.53)	1.33±0.33 (6.53)	3.11±0.91 (9.67)	4.33±0.67 (11.93)	3.67±1.67 (10.53)	1.33±0.33 (6.53)	3.11±0.91 (9.67)
3H	4.67±0.33 (12.35)	3.33±0.88 (10.34)	1.00±0.58 (4.62)	3.00±0.97 (9.10)	4.33±0.33 (11.99)	3.33±0.88 (10.34)	1.00±0.58 (4.62)	2.89±0.99 (8.98)	4.33±0.33 (11.99)	3.33±0.88 (10.34)	1.00±0.58 (4.62)	2.89±0.9 (8.98)
4H	6.67±2.91 (14.19)	3.67±0.33 (11.01)	2.00±1.00 (6.65)	4.11±1.64 (10.62)	6.67±2.91 (14.19)	2.33±1.20 (7.17)	2.00±1.00 (6.65)	3.67±1.50 (9.33)	6.67±2.91 (14.19)	2.33±1.20 (7.17)	2.00±1.00 (6.65)	3.67±1.50 (9.33)
5H	5.00±0.58 (12.87)	2.00±0.00 (8.13)	3.67±0.33 (11.01)	3.56±1.00 (10.67)	5.00±0.58 (12.87)	2.00±0.00 (8.13)	3.67±0.33 (9.26)	3.56±0.87 (10.67)	5.00±0.58 (12.87)	2.00±0.00 (8.13)	3.67±0.33 (11.01)	3.56±0.87 (10.67)
6H	7.33±0.88 (15.65)	5.67±0.67 (13.72)	0.00±0.00 (0.00)	4.33±1.06 (9.79)	7.33±0.88 (15.65)	5.67±0.67 (13.72)	0.00±0.00 (0.00)	4.33±2.22 (9.79)	7.33±0.88 (15.65)	5.67±0.67 (13.72)	0.00±0.00 (0.00)	4.33±2.22 (9.79)
7H	4.00±0.58 (11.47)	3.33±0.33 (10.49)	1.00±0.58 (4.62)	2.78±0.91 (8.86)	4.00±0.58 (11.47)	3.33±0.33 (10.49)	1.00±0.58 (4.62)	2.78±0.91 (8.86)	4.00±0.58 (11.47)	3.33±0.33 (10.49)	1.00±0.58 (4.62)	2.78±0.91 (8.86)
1NH	5.33±0.88 (13.26)	12.00±1.53 (20.19)	6.67±0.33 (14.95)	8.00±2.04 (16.13)	5.33±0.88 (13.26)	10.00±0.33 (18.74)	6.67±0.33 (14.95)	7.44±1.49 (15.65)	5.33±0.88 (13.26)	10.00±0.33 (18.41)	6.67±0.33 (14.95)	7.44±1.49 (15.65)
2NH	3.00±0.00 (9.97)	9.33±0.33 (17.78)	5.33±0.33 (13.33)	5.89±1.85 (13.69)	3.00±0.00 (9.97)	6.33±1.45 (14.39)	5.33±0.33 (13.33)	4.89±0.99 (12.56)	3.00±0.00 (9.97)	6.33±1.45 (14.39)	5.33±0.33 (13.33)	4.89±0.99 (12.56)
3NH	5.00±0.00 (12.92)	8.00±0.00 (16.42)	5.00±0.00 (12.92)	6.00±1.00 (14.08)	4.67±0.33 (12.45)	6.00±1.00 (14.07)	5.00±0.00 (12.92)	5.22±0.40 (13.15)	4.67±0.33 (12.45)	6.00±1.00 (14.07)	5.00±0.00 (12.92)	5.22±0.40 (13.15)
Mean	4.70 (11.89)	5.17 (12.13)	2.90 (8.28)	-	4.63 (11.81)	4.50 (11.38)	2.90 (8.28)	-	4.63 (11.81)	4.47 (11.34)	2.90 (8.28)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:	<u>50h</u>	<u>52h</u>	<u>54h</u>
Colony	(2.91)	(3.22)	(3.22)
Brood Cycle	(1.60)	(1.76)	(1.76)
Colony x Brood Cycle	(5.05)	(5.58)	(5.58)

Table 32. Percentage of brood cells only uncapped by various time intervals in three alternate brood cycles* starting at 68 h of treatment

Col. No.	68 h				70 h				72 h			
	Brood Cycle 1	Brood Cycle 2	Brood Cycle 3	Mean	Brood Cycle 1	Brood Cycle 2	Brood Cycle 3	Mean	Brood Cycle 1	Brood Cycle 2	Brood Cycle 3	Mean
1H	1.67±1.67 (4.31)	2.00±1.53 (6.22)	3.00±1.53 (8.15)	2.22±0.40 (6.22)	1.67±1.67 (4.31)	2.00±1.53 (6.22)	3.00±1.53 (8.15)	2.22±0.40 (6.22)	1.67±1.67 (4.31)	2.00±1.53 (6.22)	3.00±1.53 (8.15)	2.22±0.40 (6.22)
2H	4.33±0.67 (11.93)	3.67±1.67 (10.53)	1.33±0.33 (6.53)	3.11±0.91 (9.67)	4.33±0.67 (11.93)	3.67±1.67 (10.53)	1.33±0.33 (6.53)	3.11±0.91 (9.67)	4.33±0.67 (11.93)	3.67±1.67 (10.53)	1.33±0.33 (6.53)	3.11±0.91 (9.67)
3H	4.33±0.33 (11.99)	3.33±0.88 (10.34)	1.00±0.58 (4.62)	2.89±0.99 (8.98)	4.33±0.33 (11.99)	3.33±0.88 (10.34)	1.00±0.58 (4.62)	2.89±0.99 (8.98)	4.33±0.33 (11.99)	3.33±0.88 (10.34)	1.00±0.58 (4.62)	2.89±0.99 (8.98)
4H	6.67±2.91 (14.19)	2.33±1.20 (7.17)	2.00±1.00 (6.65)	3.67±1.50 (9.33)	6.67±2.91 (14.19)	2.33±1.20 (7.17)	2.00±1.00 (6.65)	3.67±1.50 (9.33)	6.67±2.91 (14.19)	2.33±1.20 (7.17)	2.00±1.00 (6.65)	3.67±1.50 (9.33)
5H	5.00±0.58 (12.87)	2.00±0.00 (8.13)	3.67±0.33 (11.01)	3.56±0.87 (10.67)	5.00±0.58 (12.87)	2.00±0.00 (8.13)	3.67±0.33 (11.01)	3.56±0.87 (10.67)	5.00±0.58 (12.87)	2.00±0.00 (8.13)	3.67±0.33 (11.01)	3.56±0.87 (10.67)
6H	7.33±0.88 (15.65)	5.67±0.67 (13.72)	0.00±0.00 (0.00)	4.33±2.22 (9.79)	7.33±0.88 (15.65)	5.67±0.67 (13.72)	0.00±0.00 (0.00)	4.33±2.22 (9.79)	7.33±0.88 (15.65)	5.67±0.67 (13.72)	0.00±0.00 (0.00)	4.33±2.22 (9.79)
7H	4.00±0.58 (11.47)	3.33±0.33 (10.49)	1.00±0.58 (4.62)	2.78±0.91 (8.86)	4.00±0.58 (11.47)	3.33±0.33 (10.49)	1.00±0.58 (4.62)	2.78±0.91 (8.86)	4.00±0.58 (11.47)	3.33±0.33 (10.49)	1.00±0.58 (4.62)	2.78±0.91 (8.86)
1NH	5.33±0.88 (13.26)	10.00±0.33 (18.41)	6.67±0.33 (14.95)	7.44±1.39 (15.64)	5.33±0.88 (13.26)	10.00±0.33 (18.41)	6.67±0.33 (14.95)	7.44±1.39 (15.64)	5.33±0.88 (13.26)	10.00±0.33 (18.41)	6.67±0.33 (14.95)	7.44±1.39 (15.64)
2NH	3.00±0.00 (9.97)	6.33±1.45 (14.39)	5.33±0.33 (13.33)	4.89±0.99 (12.56)	3.00±0.00 (9.97)	6.33±1.45 (14.39)	5.33±0.33 (13.33)	4.89±0.99 (12.56)	3.00±0.00 (9.97)	6.33±1.45 (14.39)	5.33±0.33 (13.33)	4.89±0.99 (12.56)
3NH	4.67±0.33 (12.45)	6.00±1.00 (14.07)	5.00±0.00 (12.92)	5.22±0.40 (13.15)	4.67±0.33 (12.45)	6.00±1.00 (14.07)	5.00±0.00 (12.92)	5.22±0.40 (13.15)	4.67±0.33 (12.45)	6.00±1.00 (14.07)	5.00±0.00 (12.92)	5.22±0.40 (13.15)
Mean	4.63 (11.81)	4.47 (11.34)	2.90 (8.28)	-	4.63 (11.81)	4.47 (11.34)	2.90 (8.28)	-	4.63 (11.81)	4.47 (11.34)	2.90 (8.28)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

	<u>68h</u>	<u>70h</u>	<u>72h</u>
Colony	(3.22)	(3.22)	(3.22)
Brood Cycle	(1.76)	(1.76)	(1.76)
Colony x Brood Cycle	(5.58)	(5.58)	(5.58)

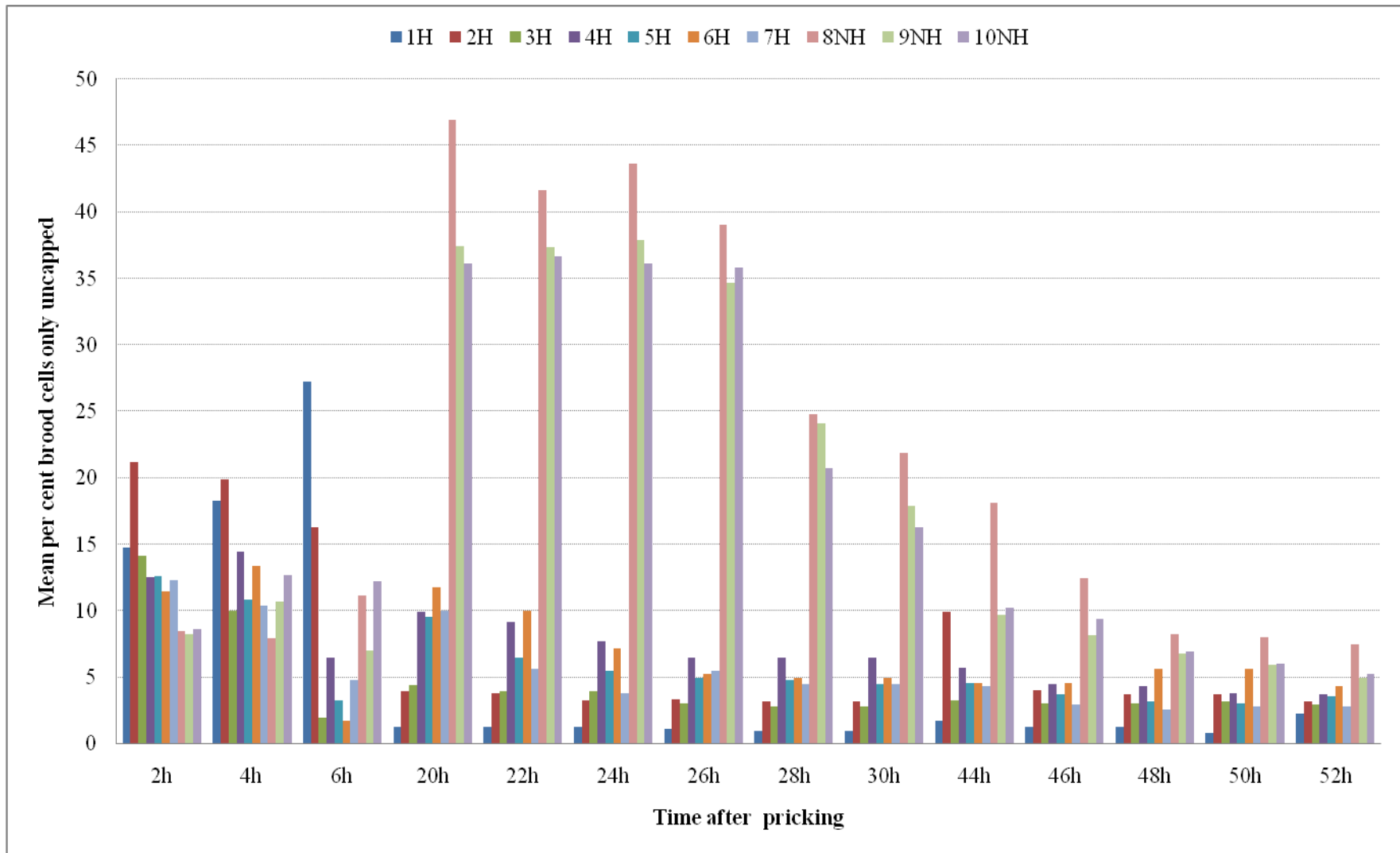


Fig. 23 Mean percentage of brood cells only uncapped by various intervals after pricking in three alternate brood cycles

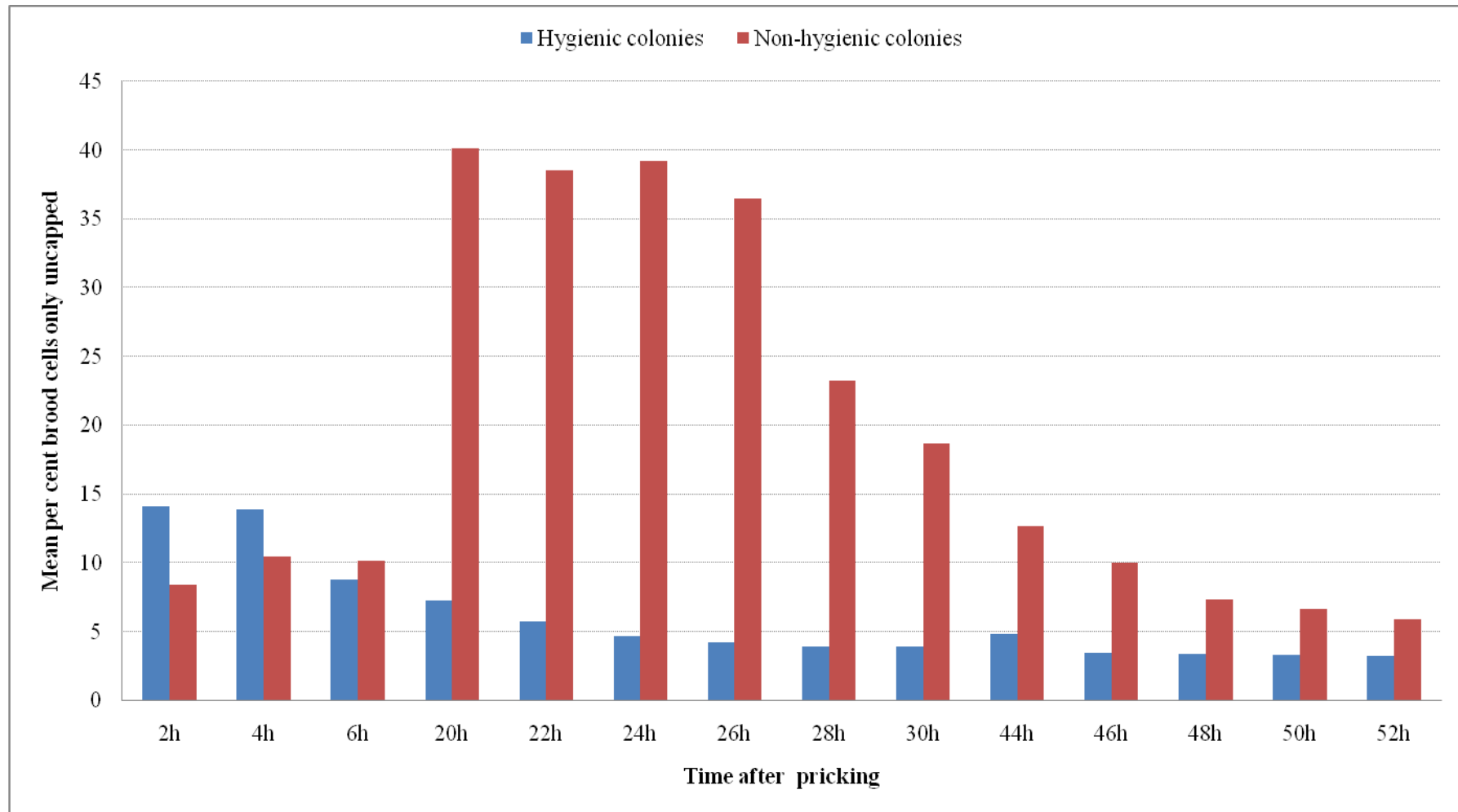


Fig. 24. Mean percentage of brood cells only uncapped by various time intervals after pricking in three alternate brood cycles in hygienic and non-hygienic colonies

Table 33. Percentage of brood cells remaining capped by various time intervals after pricking in three alternate brood cycles*

Col. No.	2 h				4 h				6 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	88.00±0.58 (69.71)	82.67±1.45 (65.41)	83.00±0.58 (65.63)	84.56±1.72 (66.92)	66.67±1.20 (54.72)	76.33±0.88 (60.87)	75.33±0.33 (60.20)	72.78±3.07 (58.60)	54.00±0.58 (47.28)	56.67±1.45 (48.81)	57.00±0.58 (49.00)	55.89±0.95 (48.37)
2H	80.33±1.45 (63.68)	77.00±0.58 (61.32)	78.00±0.58 (62.01)	78.44±0.99 (62.34)	76.67±0.88 (61.10)	72.33±0.33 (58.24)	71.00±0.58 (57.40)	73.33±1.71 (58.91)	70.67±0.67 (57.19)	63.33± (52.71)	59.00±0.58 (34.43)	64.33±3.41 (53.36)
3H	89.00±0.58 (70.61)	83.67±0.67 (66.14)	85.00±0.58 (67.19)	85.89±1.60 (67.98)	83.00±1.53 (65.66)	80.67±0.67 (63.90)	80.00±0.00 (63.41)	81.22±0.91 (64.32)	77.67±1.45 (61.80)	79.33±0.67 (62.94)	79.00±0.58 (62.70)	78.67±0.51 (62.48)
4H	89.67±0.33 (71.22)	86.33±0.88 (68.30)	86.00±0.00 (68.00)	87.33±1.17 (69.17)	83.67±0.88 (66.15)	82.33±1.20 (65.14)	81.33±0.33 (64.38)	82.44±0.68 (65.22)	78.33±1.20 (62.25)	78.33±0.88 (62.24)	77.67±0.33 (61.77)	78.11±0.22 (62.09)
5H	86.33±0.67 (68.29)	89.00±0.58 (70.61)	87.00±0.58 (68.85)	87.44±0.80 (69.25)	85.00±1.15 (67.22)	86.67±0.67 (68.57)	84.00±0.58 (66.40)	85.22±0.78 (67.40)	81.00±0.58 (64.14)	82.67±0.33 (65.37)	79.67±0.33 (63.17)	81.11±0.87 (64.23)
6H	87.67±0.88 (69.44)	90.00±0.58 (71.55)	88.00±0.58 (69.71)	88.56±0.73 (70.23)	83.67±0.88 (66.15)	84.33±1.20 (66.69)	81.33±0.33 (64.38)	83.11±0.91 (65.74)	79.00±0.58 (62.70)	80.67±0.67 (62.70)	78.00±0.00 (62.00)	79.22±0.78 (62.87)
7H	88.33±0.88 (70.03)	88.67±0.67 (70.31)	86.00±0.58 (68.01)	87.67±0.84 (69.45)	84.33±0.88 (66.67)	86.00±2.00 (68.11)	81.00±0.58 (64.14)	83.78±1.47 (66.31)	77.67±0.88 (61.78)	86.67±0.67 (68.57)	75.00±0.00 (59.98)	79.78±3.53 (63.44)
1NH	90.67±0.88 (72.22)	94.33±0.67 (76.24)	87.67±0.88 (71.25)	91.56±1.93 (73.24)	87.67±0.33 (69.42)	90.67±1.86 (72.34)	87.67±0.88 (69.44)	88.67±1.00 (70.40)	84.00±0.58 (66.40)	87.67±2.33 (69.57)	73.00±1.00 (58.68)	81.56±4.41 (64.88)
2NH	88.00±1.00 (69.73)	96.67±1.20 (79.90)	90.67±0.67 (72.20)	91.78±2.56 (73.95)	86.00±0.58 (68.01)	93.00±0.58 (74.65)	82.33±1.45 (65.16)	87.11±3.13 (69.27)	83.00±0.58 (65.63)	89.33±0.67 (70.93)	78.33±0.88 (62.24)	83.56±3.19 (66.27)
3NH	90.00±0.58 (71.55)	89.00±0.58 (70.61)	95.33±1.45 (77.84)	91.44±1.96 (73.33)	89.33±0.67 (70.93)	89.67±0.33 (71.22)	75.33±0.67 (60.20)	84.78±4.72 (67.45)	86.33±0.88 (68.30)	87.00±0.58 (68.85)	67.00±1.73 (54.93)	80.11±6.56 (64.02)
Mean	87.80 (69.65)	87.33 (70.05)	86.87 (69.07)	-	82.60 (65.60)	84.20 (66.97)	79.93 (63.51)	-	77.17 (61.75)	79.17 (63.39)	72.37 (58.47)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:	<u>2h</u>	<u>4h</u>	<u>6h</u>
Colony	(1.36)	(1.23)	(1.06)
Brood Cycle	(0.75)	(0.67)	(0.58)
Colony x Brood Cycle	(2.36)	(2.13)	(1.84)

Table 34. Percentage of brood cells remaining capped by various time intervals after pricking in three alternate brood cycles* starting at after 20 h of treatment

Col. No.	20 h				22 h				24 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	7.33±0.33 (15.70)	8.67±0.33 (17.11)	8.67±0.33 (17.11)	8.22±0.45 (16.64)	5.33±0.33 (13.33)	6.33±0.33 (14.56)	7.33±0.33 (15.70)	6.33±0.58 (14.53)	3.33±0.33 (10.49)	4.00±0.00 (11.53)	5.00±0.58 (12.87)	4.11±0.49 (11.63)
2H	1.00±0.58 (4.62)	3.33±0.67 (10.40)	4.00±0.58 (11.47)	2.78±0.91 (8.83)	0.67±0.33 (3.82)	2.67±0.33 (9.36)	3.33±0.33 (10.49)	2.22±0.80 (7.89)	0.33±0.33 (1.91)	1.67±0.33 (7.33)	2.67±0.33 (9.36)	1.56±0.68 (6.20)
3H	1.67±0.88 (6.03)	2.00±0.58 (7.94)	4.00±0.58 (11.47)	2.56±0.73 (8.48)	1.33±0.58 (5.42)	2.00±0.58 (7.94)	3.00±0.33 (9.97)	2.11±6.30 (7.78)	1.33±0.33 (5.42)	2.00±0.58 (7.94)	3.00±0.67 (9.97)	2.11±0.49 (7.78)
4H	6.00±1.53 (13.97)	5.67±0.67 (13.72)	6.67±0.67 (14.92)	6.11±0.29 (14.20)	3.33±0.88 (10.34)	5.67±0.00 (13.72)	6.00±0.00 (14.17)	5.00±0.84 (12.74)	2.67±0.33 (9.36)	4.00±1.00 (11.32)	4.67±0.67 (12.41)	3.78±0.59 (11.03)
5H	11.33±0.67 (19.65)	8.33±0.88 (16.73)	7.00±1.00 (15.25)	8.89±1.28 (17.21)	11.33± (19.65)	7.67±1.20 (15.98)	5.67±0.33 (13.75)	8.22±1.66 (16.46)	9.33±0.67 (17.76)	7.33±1.45 (15.56)	4.67±0.67 (12.41)	7.11±1.35 (15.24)
6H	4.33±0.33 (11.99)	6.00± (14.17)	8.67±0.88 (17.07)	7.67±1.26 (14.41)	0.00±0.00 (0.00)	2.67±0.33 (9.36)	6.67±1.20 (14.85)	3.11±1.94 (8.07)	0.00±0.00 (0.00)	2.00±0.00 (8.13)	6.67±1.20 (14.85)	2.89±1.98 (7.66)
7H	7.00±0.00 (15.34)	8.33± (16.75)	3.33±0.88 (10.34)	6.22±1.50 (14.14)	4.00±0.58 (11.47)	3.67±0.33 (11.01)	2.67±0.33 (9.36)	3.44±0.40 (10.61)	4.00±0.58 (11.47)	3.67±0.33 (11.01)	2.33±0.33 (8.74)	3.33±0.51 (10.41)
1NH	19.33±1.45 (26.04)	16.33±0.88 (23.81)	24.00±0.58 (29.32)	19.89±2.23 (26.39)	16.33±0.88 (23.81)	14.33±0.67 (22.23)	21.00±0.58 (27.26)	17.22±1.98 (24.43)	10.67±0.88 (19.02)	13.33±0.33 (21.41)	14.33±0.33 (22.26)	12.78±1.09 (20.89)
2NH	19.33±2.40 (25.99)	24.00±0.00 (29.32)	22.00±0.58 (27.96)	21.71±1.35 (27.76)	17.67±2.03 (24.77)	21.00±0.58 (27.56)	17.00±0.58 (24.33)	18.56±1.24 (25.46)	13.33±1.45 (21.30)	21.00±0.67 (27.26)	11.00±0.67 (19.35)	15.11±3.02 (22.64)
3NH	18.33±0.88 (25.33)	22.33±0.67 (28.19)	16.33±0.33 (23.83)	19.00±1.76 (25.78)	16.00±0.58 (23.56)	21.67±1.33 (27.71)	10.67±0.33 (19.05)	16.11±3.18 (23.44)	9.67±0.88 (18.07)	15.33±1.00 (23.04)	9.33±0.58 (17.78)	11.44±1.95 (19.63)
Mean	9.57 (16.47)	10.50 (17.81)	10.47 (17.87)	-	7.37 (13.62)	9.17 (15.91)	8.77 (15.89)	-	5.47 (11.48)	7.43 (14.45)	6.37 (14.00)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

	<u>20h</u>	<u>22h</u>	<u>24h</u>
Colony	(1.78)	(1.54)	(1.64)
Brood Cycle	(0.98)	(0.84)	(0.90)
Colony x Brood Cycle	(3.09)	(2.67)	(2.83)

Col. No.	26 h				28 h				30 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	1.67±0.33 (7.33)	3.00±0.00 (9.97)	3.67±0.33 (11.01)	2.78±0.59 (9.44)	1.67±0.33 (7.33)	3.00±0.00 (9.97)	3.67±0.33 (11.01)	2.78±0.59 (9.44)	1.67±0.33 (7.33)	3.00±0.00 (9.97)	3.67±0.33 (11.01)	2.78±0.59 (9.44)
2H	0.33±0.33 (1.91)	1.00±0.58 (4.62)	1.33±0.33 (6.53)	0.89±0.29 (4.36)	0.33±0.33 (1.91)	1.00±0.58 (4.62)	1.33±0.33 (6.53)	0.89±0.29 (4.36)	0.33±0.33 (1.91)	1.00±0.58 (4.62)	1.33±0.33 (6.53)	0.89±0.29 (4.36)
3H	0.67±0.67 (2.71)	2.00±0.58 (7.95)	2.00±0.00 (8.13)	1.56±0.44 (6.26)	0.33±0.33 (1.91)	1.33±0.33 (6.53)	1.00±0.00 (5.74)	0.89±0.29 (4.36)	0.33±0.33 (1.91)	1.33±0.33 (6.53)	1.00±0.00 (5.74)	0.89±0.29 (4.73)
4H	2.67±1.45 (7.63)	4.00±1.00 (11.32)	4.67±0.67 (12.41)	3.78±0.59 (10.45)	2.67±1.45 (7.63)	4.00±1.00 (11.32)	4.67±0.67 (12.41)	3.78±0.59 (10.45)	2.67±1.45 (7.63)	4.00±1.00 (11.32)	4.67±0.67 (12.41)	3.78±0.59 (10.45)
5H	9.00±0.58 (17.43)	6.00±1.00 (14.08)	4.00±0.00 (11.53)	6.33±1.45 (14.35)	5.00±0.58 (12.87)	6.00±1.00 (14.08)	4.00±0.00 (11.53)	5.00±0.58 (12.83)	5.00±0.58 (12.87)	6.00±1.00 (14.08)	4.00±0.00 (11.53)	5.00±0.58 (12.83)
6H	0.00±0.00 (0.00)	1.33±0.67 (5.42)	5.33±0.33 (13.33)	2.22±1.60 (6.25)	0.00±0.00 (0.00)	1.33±0.67 (5.42)	5.33±0.33 (13.33)	2.22±1.60 (6.25)	0.00±0.00 (0.00)	1.33±0.67 (5.42)	5.33±0.33 (13.33)	2.22±1.60 (6.25)
7H	4.00±0.58 (11.47)	3.67±0.33 (11.01)	2.33±0.33 (8.74)	3.33±0.51 (10.41)	4.00±0.58 (11.47)	3.67±0.33 (11.01)	2.33±0.33 (8.74)	3.33±0.51 (10.41)	4.00±0.58 (11.47)	3.67±0.33 (11.01)	2.33±0.33 (8.74)	3.33±0.51 (10.41)
1NH	10.67±0.88 (19.02)	13.00±0.00 (21.13)	7.00±0.58 (15.31)	10.22±1.72 (18.49)	6.67±0.88 (14.89)	9.33±0.67 (17.76)	7.00±0.58 (15.31)	7.67±0.84 (15.99)	6.67±0.88 (14.89)	9.33±0.67 (17.76)	6.00±1.00 (14.08)	7.33±1.02 (15.58)
2NH	11.33±1.33 (19.61)	15.00±0.58 (22.77)	7.33±0.33 (15.70)	11.22±2.21 (19.36)	9.00±1.15 (17.38)	10.67±0.88 (19.02)	7.33±0.33 (15.70)	9.00±0.96 (17.37)	5.67±0.88 (13.68)	9.00±0.00 (17.45)	6.67±0.33 (14.95)	7.11±0.99 (15.36)
3NH	10.33±0.33 (18.74)	8.00±1.00 (16.37)	4.33±1.67 (11.36)	7.56±1.75 (15.49)	8.00±0.58 (16.40)	8.00±1.00 (16.37)	5.67 (13.72)	7.22±0.78 (15.50)	8.00±0.58 (16.40)	8.00±1.00 (16.37)	5.67±0.67 (13.72)	7.22±0.78 (15.50)
Mean	5.06 (10.59)	5.70 (12.46)	4.20 (11.41)	-	3.77 (9.18)	4.83 (11.61)	4.23 (11.40)	-	3.43 (8.81)	4.67 (11.45)	4.07 (11.21)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

	<u>26h</u>	<u>28h</u>	<u>30h</u>
Colony	(2.33)	(2.13)	(2.13)
Brood Cycle	(1.27)	(1.16)	(1.17)
Colony x Brood Cycle	(4.03)	(3.68)	(3.69)

Table 35. Percentage of brood cells remaining capped by various time intervals after pricking in three alternate brood cycles* starting at 44 h of treatment

Col. No.	44 h				46 h				48 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	1.33±0.67 (5.42)	2.67±1.45 (7.63)	0.00±0.00 (0.00)	1.33±0.77 (4.35)	1.33±0.67 (5.42)	2.67±1.45 (7.63)	0.00±0.00 (0.00)	1.33±0.77 (4.35)	1.33±0.67 (5.42)	0.67±0.33 (2.71)	0.00±0.00 (0.00)	0.67±0.38 (2.71)
2H	0.00±0.00 (0.00)	1.00±0.58 (4.62)	1.33±0.67 (5.42)	0.78±0.40 (3.35)	0.00±0.00 (0.00)	1.00±0.58 (4.62)	1.33±0.67 (5.42)	0.78±0.40 (3.35)	0.00±0.00 (0.00)	0.67±0.33 (2.71)	0.00±0.00 (0.00)	0.22±0.22 (0.90)
3H	0.67±0.67 (2.71)	1.33±0.67 (6.53)	1.00±0.58 (5.73)	1.00±0.19 (4.99)	0.67±0.67 (2.71)	1.33±0.67 (5.42)	1.00±0.58 (4.62)	1.00±0.19 (4.62)	0.33±0.33 (1.92)	1.33±0.67 (6.52)	1.00±0.58 (5.73)	0.89±0.29 (4.72)
4H	2.67±1.45 (7.63)	4.00±1.00 (11.32)	4.67±0.67 (12.41)	3.78±0.59 (10.45)	2.00±0.58 (7.94)	3.33±0.88 (10.34)	4.67±0.67 (12.41)	3.33±0.77 (10.34)	2.00±0.58 (6.64)	2.33±0.88 (8.74)	3.00±0.00 (9.97)	2.44±0.29 (8.45)
5H	5.00±0.58 (12.87)	6.00±1.00 (14.08)	4.00±0.00 (11.53)	5.00±0.58 (12.83)	5.00±0.58 (12.87)	6.00±1.00 (14.08)	4.00±0.00 (11.53)	5.00±0.58 (12.83)	3.67±0.33 (11.01)	6.00±1.00 (14.08)	4.00±0.00 (11.53)	4.56±0.73 (12.20)
6H	0.00±0.00 (0.00)	1.33±0.67 (5.42)	5.00±0.00 (12.92)	2.11±1.50 (6.11)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	5.00±0.00 (12.92)	1.67±1.67 (4.31)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	5.00±0.00 (12.92)	1.67±1.67 (4.30)
7H	3.00±0.00 (9.97)	4.33±0.33 (11.99)	2.33±0.33 (8.74)	3.22±0.59 (10.23)	1.67±1.67 (7.33)	4.33±0.67 (11.99)	2.33±0.33 (8.74)	2.78±0.80 (9.33)	1.67±1.67 (7.33)	4.33±0.67 (11.99)	2.33±0.33 (8.74)	2.78±0.80 (9.35)
1NH	4.67±0.33 (12.45)	9.33±0.67 (17.76)	6.00±1.00 (14.08)	6.67±1.39 (14.77)	4.67±0.33 (12.45)	8.67±0.67 (12.45)	6.00±1.00 (14.08)	6.44±1.18 (14.54)	4.00±1.00 (11.32)	7.67±0.33 (16.07)	6.00±1.00 (14.08)	5.89±1.06 (13.82)
2NH	4.67±0.67 (12.41)	9.00±0.00 (17.45)	6.67±0.33 (14.95)	6.78±1.25 (14.94)	4.67±0.67 (12.41)	7.33±0.33 (15.70)	6.67±0.33 (14.95)	6.22±0.80 (14.35)	4.33±0.33 (11.99)	6.67±0.88 (14.89)	6.67±0.33 (14.95)	5.89±0.78 (13.82)
3NH	5.67±0.67 (13.72)	9.00±0.58 (17.43)	5.67±0.67 (13.72)	6.78±1.11 (14.96)	5.67±0.67 (13.72)	8.67±0.67 (17.09)	5.67±0.67 (13.72)	6.67±1.00 (14.85)	2.00±0.00 (7.94)	9.00±0.00 (17.45)	5.67±0.67 (13.72)	5.56±2.02 (13.10)
Mean	2.67 (7.72)	4.77 (11.39)	3.67 (9.95)	-	2.57 (7.49)	4.37 (10.54)	3.67 (9.95)	-	1.93 (6.38)	3.87 (9.63)	3.37 (9.17)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:

	44h	46h	48h
Colony	(2.62)	(2.23)	(1.96)
Brood Cycle	(1.43)	(1.22)	(1.07)
Colony x Brood Cycle	(4.53)	(3.87)	(3.39)

Col. No.	50 h				52 h				54 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	1.33±0.67 (5.42)	0.67±0.67 (3.82)	0.00±0.00 (0.00)	0.67±0.38 (3.08)	1.33±0.67 (5.42)	0.67±0.67 (3.82)	0.00±0.00 (0.00)	0.67±0.38 (3.08)	1.33±0.67 (5.42)	0.67±0.67 (3.82)	0.00±0.00 (0.00)	0.67±0.38 (3.08)
2H	0.00±0.00 (0.00)	0.67±0.58 (2.71)	0.00±0.00 (0.00)	0.22±0.22 (0.90)	0.00±0.00 (0.00)	0.67±0.58 (2.71)	0.00±0.00 (0.00)	0.22±0.22 (0.90)	0.00±0.00 (0.00)	0.67±0.58 (2.71)	0.00±0.00 (0.00)	0.22±0.22 (0.90)
3H	0.33±0.33 (1.91)	1.33±0.67 (6.53)	1.00±0.58 (5.74)	0.89±0.29 (4.73)	0.33±0.33 (1.91)	1.33±0.67 (6.53)	1.00±0.58 (5.74)	0.89±0.29 (4.73)	0.33±0.33 (1.91)	1.33±0.67 (6.53)	1.00±0.58 (5.74)	0.89±0.29 (4.73)
4H	2.00±0.58 (6.65)	2.33±0.88 (8.74)	3.00±0.33 (9.97)	2.44±0.29 (8.45)	2.00±0.58 (6.65)	2.33±0.88 (8.74)	3.00±0.33 (9.97)	2.44±0.29 (8.45)	2.00±0.58 (6.65)	2.33±0.88 (8.74)	3.00±0.33 (9.97)	2.44±0.29 (8.45)
5H	3.67±0.33 (11.01)	6.00±1.00 (14.07)	4.00±0.00 (11.53)	4.56±0.73 (12.20)	3.67±0.33 (11.01)	6.00±1.00 (14.07)	4.00±0.00 (11.53)	4.56±0.73 (12.20)	3.67±0.33 (11.01)	6.00±1.00 (14.07)	4.00±0.00 (11.53)	4.56±0.73 (12.20)
6H	0.00±0.00 (0.00)	0.00±0.00 (0.00)	5.00±0.00 (12.92)	1.67±1.67 (4.31)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	5.00±0.00 (12.92)	1.67±1.67 (4.31)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	5.00±0.00 (12.92)	1.67±1.67 (4.31)
7H	1.67±1.67 (7.33)	4.33±0.67 (11.99)	2.33±0.33 (8.74)	2.78±0.80 (9.35)	1.67±1.67 (7.33)	4.33±0.67 (11.99)	2.33±0.33 (8.74)	2.78±0.80 (9.35)	1.67±1.67 (7.33)	4.33±0.67 (11.99)	2.33±0.33 (8.74)	2.78±0.80 (9.35)
1NH	4.00±1.00 (11.32)	7.67±0.33 (16.06)	6.00±1.00 (14.08)	5.89±1.06 (13.82)	4.00±1.00 (11.32)	7.67±0.33 (16.06)	6.00±1.00 (14.08)	5.89±1.06 (13.82)	4.00±1.00 (11.32)	7.67±0.33 (16.06)	6.00±1.00 (14.08)	5.89±1.06 (13.82)
2NH	4.33±0.33 (11.99)	6.67±0.88 (14.89)	6.67±0.33 (14.95)	5.89±0.78 (13.94)	4.33±0.33 (11.99)	6.67±0.88 (14.89)	6.67±0.33 (14.95)	5.89±0.78 (13.94)	4.33±0.33 (11.99)	6.67±0.88 (14.89)	6.67±0.33 (14.95)	5.89±0.78 (13.94)
3NH	2.00±0.33 (8.13)	7.67±0.33 (16.06)	5.67±0.67 (13.72)	5.11±1.66 (12.64)	2.00±0.33 (8.13)	7.67±0.33 (16.06)	5.67±0.67 (13.72)	5.11±1.66 (12.64)	2.00±0.33 (8.13)	7.67±0.33 (16.06)	5.67±0.67 (13.72)	5.11±1.66 (12.64)
Mean	1.93 (6.38)	3.73 (9.49)	3.37 (9.17)	-	1.93 (6.38)	3.73 (9.49)	3.37 (9.17)	-	1.93 (6.38)	3.73 (9.49)	3.37 (9.17)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:	<u>50h</u>	<u>52h</u>	<u>54h</u>
Colony	(1.96)	(1.96)	(1.96)
Brood Cycle	(1.08)	(1.08)	(1.08)
Colony x Brood Cycle	(3.40)	(3.40)	(3.40)

Table 36. Percentage of brood cells remaining capped by various time intervals after pricking in three alternate brood cycles* starting at 68 h of treatment

Col. No.	68 h				70 h				72 h			
	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean	Brood Cycle I	Brood Cycle II	Brood Cycle III	Mean
1H	1.33±0.67 (5.42)	0.67±0.67 (3.82)	0.00±0.00 (0.00)	0.67±0.38 (3.08)	1.33±0.67 (5.42)	0.67±0.67 (3.82)	0.00±0.00 (0.00)	0.67±0.38 (3.08)	1.33±0.67 (5.42)	0.67±0.67 (3.82)	0.00±0.00 (0.00)	0.67±0.38 (3.08)
2H	0.00±0.00 (0.00)	0.67±0.58 (2.71)	0.00±0.00 (0.00)	0.22±0.22 (0.90)	0.00±0.00 (0.00)	0.67±0.58 (2.71)	0.00±0.00 (0.00)	0.22±0.22 (0.90)	0.00±0.00 (0.00)	0.67±0.58 (2.71)	0.00±0.00 (0.00)	0.22±0.22 (0.90)
3H	0.33±0.33 (1.91)	1.33±0.67 (6.53)	1.00±0.58 (5.74)	0.89±0.29 (4.73)	0.33±0.33 (1.91)	1.33±0.67 (6.53)	1.00±0.58 (5.74)	0.89±0.29 (4.73)	0.33±0.33 (1.91)	1.33±0.67 (6.53)	1.00±0.58 (5.74)	0.89±0.29 (4.73)
4H	2.00±0.58 (6.65)	2.33±0.88 (8.74)	3.00±0.33 (9.97)	2.44±0.29 (8.45)	2.00±0.58 (6.65)	2.33±0.88 (8.74)	3.00±0.33 (9.97)	2.44±0.29 (8.45)	2.00±0.58 (6.65)	2.33±0.88 (8.74)	3.00±0.33 (9.97)	2.44±0.29 (8.45)
5H	3.67±0.33 (11.01)	6.00±1.00 (14.07)	4.00±0.00 (11.53)	4.56±0.73 (12.20)	3.67±0.33 (11.01)	6.00±1.00 (14.07)	4.00±0.00 (11.53)	4.56±0.73 (12.20)	3.67±0.33 (11.01)	6.00±1.00 (14.07)	4.00±0.00 (11.53)	4.56±0.73 (12.20)
6H	0.00±0.00 (0.00)	0.00±0.00 (0.00)	5.00±0.00 (12.92)	1.67±1.67 (4.31)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	5.00±0.00 (12.92)	1.67±1.67 (4.31)	0.00±0.00 (0.00)	0.00±0.00 (0.00)	5.00±0.00 (12.92)	1.67±1.67 (4.31)
7H	1.67±1.67 (7.33)	4.33±0.67 (11.99)	2.33±0.33 (8.74)	2.78±0.80 (9.35)	1.67±1.67 (7.33)	4.33±0.67 (11.99)	2.33±0.33 (8.74)	2.78±0.80 (9.35)	1.67±1.67 (7.33)	4.33±0.67 (11.99)	2.33±0.33 (8.74)	2.78±0.80 (9.35)
1NH	4.00±1.00 (11.32)	7.67±0.33 (16.06)	6.00±1.00 (14.08)	5.89±1.06 (13.82)	4.00±1.00 (11.32)	7.67±0.33 (16.06)	6.00±1.00 (14.08)	5.89±1.06 (13.82)	4.00±1.00 (11.32)	7.67±0.33 (16.06)	6.00±1.00 (14.08)	5.89±1.06 (13.82)
2NH	4.33±0.33 (11.99)	6.67±0.88 (14.89)	6.67±0.33 (14.95)	5.89±0.78 (13.94)	4.33±0.33 (11.99)	6.67±0.88 (14.89)	6.67±0.33 (14.95)	5.89±0.78 (13.94)	4.33±0.33 (11.99)	6.67±0.88 (14.89)	6.67±0.33 (14.95)	5.89±0.78 (13.94)
3NH	2.00±0.33 (8.13)	7.67±0.33 (16.06)	5.67±0.67 (13.72)	5.11±1.66 (12.64)	2.00±0.33 (8.13)	7.67±0.33 (16.06)	5.67±0.67 (13.72)	5.11±1.66 (12.64)	2.00±0.33 (8.13)	7.67±0.33 (16.06)	5.67±0.67 (13.72)	5.11±1.66 (12.64)
Mean	1.93 (6.38)	3.73 (9.49)	3.37 (9.17)	-	1.93 (6.38)	3.73 (9.49)	3.37 (9.17)	-	1.93 (6.38)	3.73 (9.49)	3.37 (9.17)	-

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage transformation}}$

*6 weeks interval between each of the two consecutive test brood cycles

LSD (p=0.05) for:	<u>68h</u>	<u>70h</u>	<u>72h</u>
Colony	(1.96)	(1.96)	(1.96)
Brood Cycle	(1.08)	(1.08)	(1.08)
Colony x Brood Cycle	(3.40)	(3.40)	(3.40)

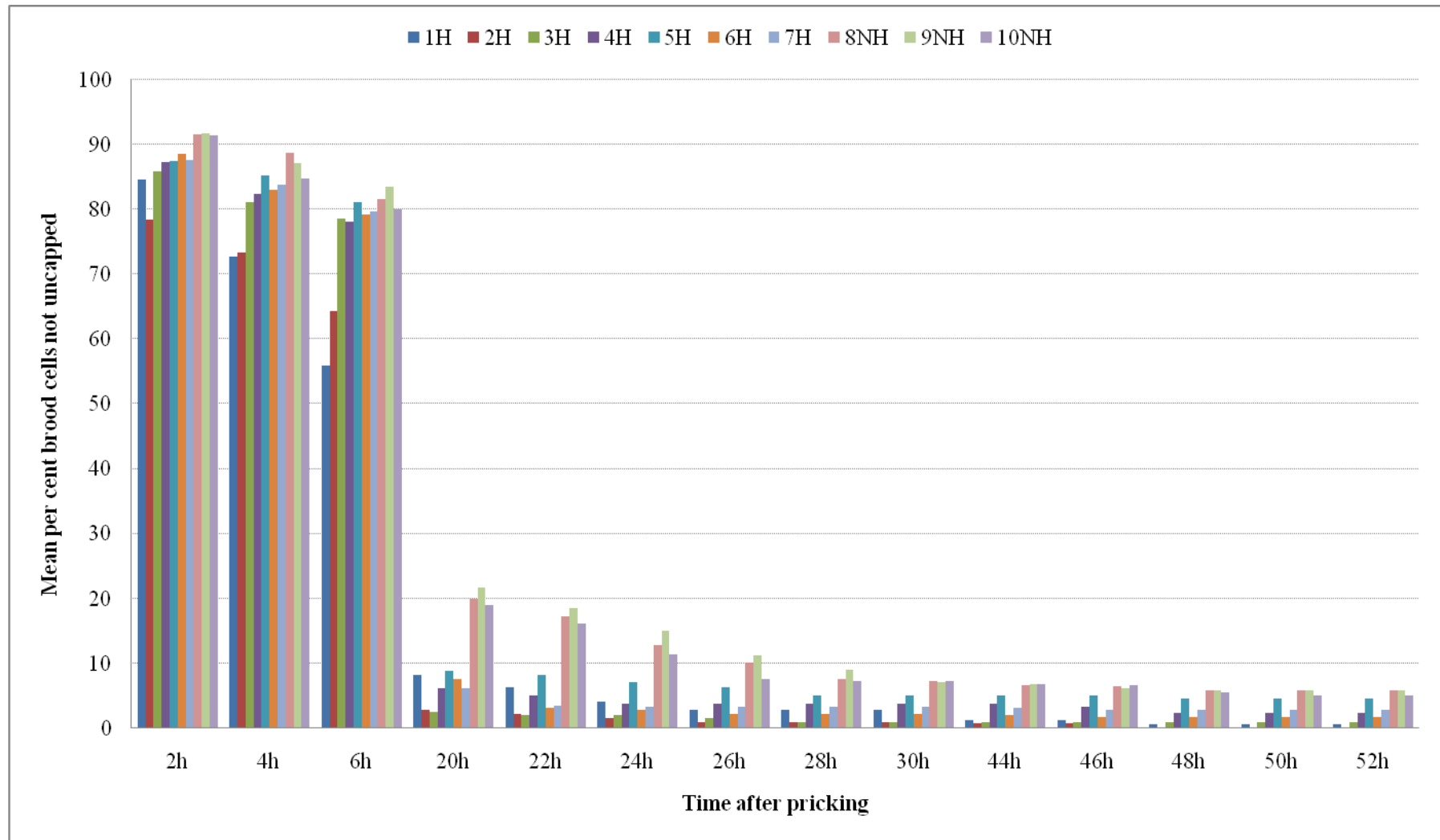


Fig. 25 Mean percentage of brood cells remaining capped by various time intervals after pricking in three alternate brood cycles

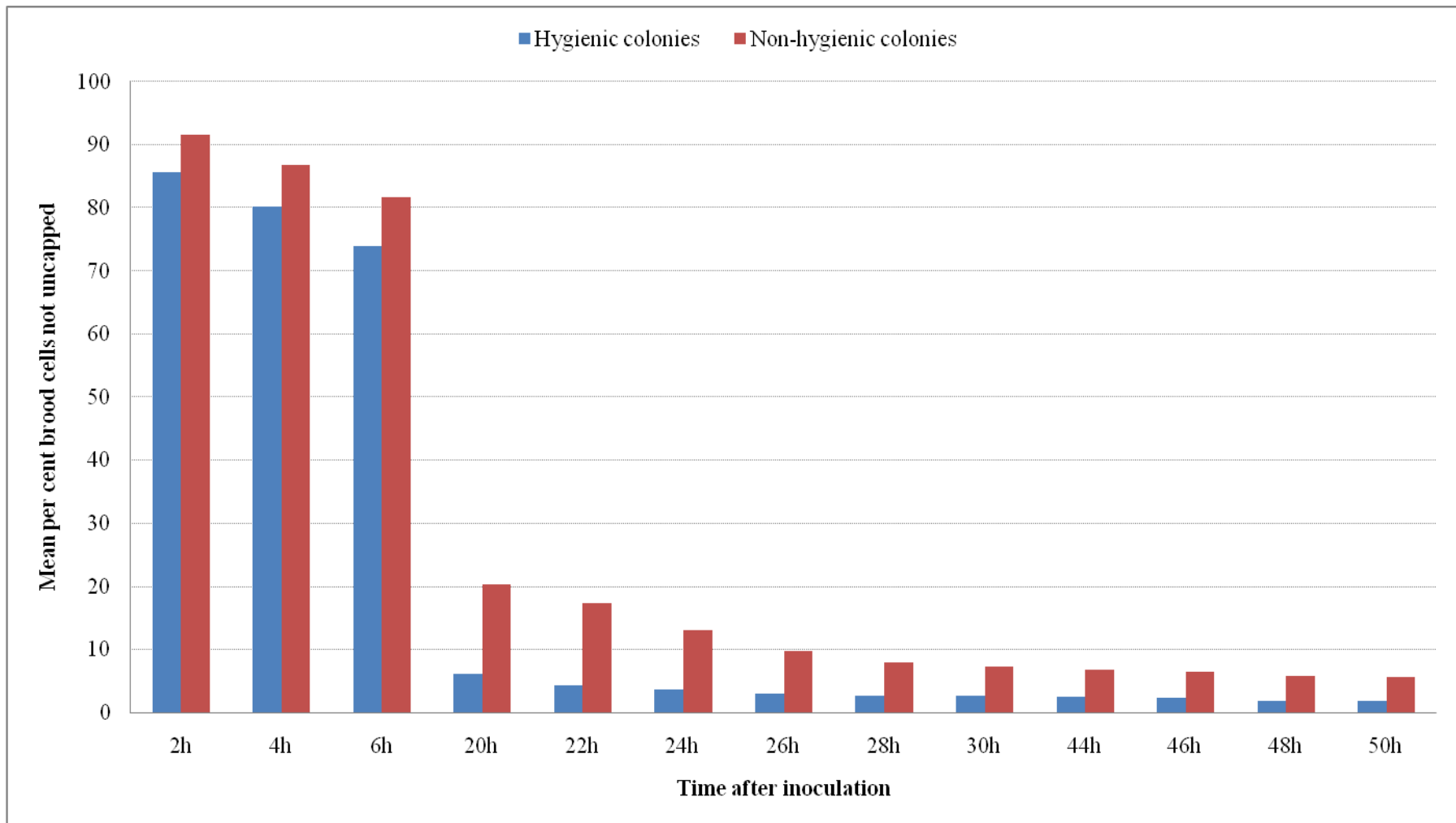


Fig. 26 Mean percentage of brood cells remaining capped by various time intervals after pricking in three alternate brood cycles in hygienic and non-hygienic colonies

4.7 Validation of markers identified to be associated with high hygienic behaviour of *A. mellifera* colonies

An experiment was conducted to validate the five SSR markers reported to be linked with the hygienic behaviour using the queens of the selected 10 colonies (7 hygienic and 3 non-hygienic). The wing clip from each of the queen bees of the 10 selected (7 hygienic and 3 non-hygienic colonies) were taken for DNA extraction, thus non-destructive sampling of queen bee was done; for genotyping with the five SSR markers reported to be linked with hygienic behaviour (Oxley *et al* 2010). The PCR amplification showed that no variation was observed for the markers among the queens, representing the hygienic and the non-hygienic colonies (Fig. 27).

4.8 Validation of screened hygienic colonies against *V. destructor* infestation

Varroa mites were used in inoculating worker larval brood near capping. Single mite was inoculated in each brood cell. The experiment was conducted during autumn and spring breeding seasons. In total, 21 inoculations (three groups of seven brood cells each) were made in every test colony, thrice in succession. The total time period between inoculation and brood removal was also recorded.

4.8.1 Validation during autumn season, 2016

Observations were recorded at every 2 h interval since inoculations till the complete removal of mite infested brood.

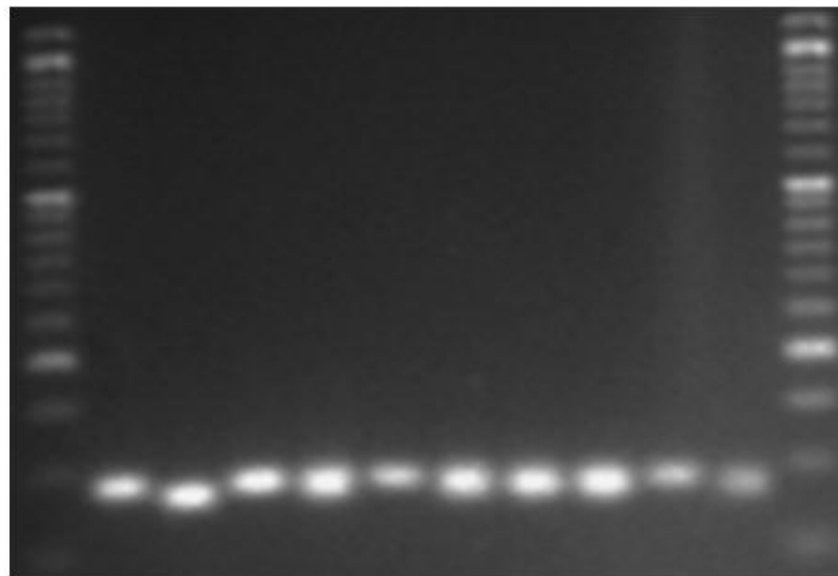
4.8.1.1 *V. destructor* inoculated brood removal

In the seven hygienic colonies, the mean of *Varroa* mite inoculated brood cells emptied after 2, 4 and 6 h was 1.36 (range between 0.00-0.3.19%); 3.17 (range: 1.57-4.76%) and 5.66 per cent (range:4.76-9.52%), respectively. In the non-hygienic colonies, the mean was 0, 0.52 (range: 0.00-1.57%) and 2.11 per cent (range: 1.57- 3.19%) after 2, 4 and 6 h, respectively (Table 37, Fig. 28 and 29).

Among the four of the hygienic colonies (2H, 3H, 7H and 6H), mean brood removal ranged between 85.71- 88.90 per cent after 20 h of inoculation while among the non-hygienic colonies at this hour, it was in the range of 42.86-58.71 per cent. After 24 h of the inoculations, for the 7 selected hygienic colonies, a mean of 93.43 per cent of brood removal was recorded (range: 85.71-100%), while for the three non-hygienic colonies, the mean brood removal was only 61.90 per cent (range: 53.95-69.86%). By 30 h after inoculation, six hygienic colonies had achieved 100 per cent emptiness of the inoculated brood cells and in non-hygienic colonies, it was in the range of 71.83-85.71 per cent (Table 38, Fig. 28 and 29).

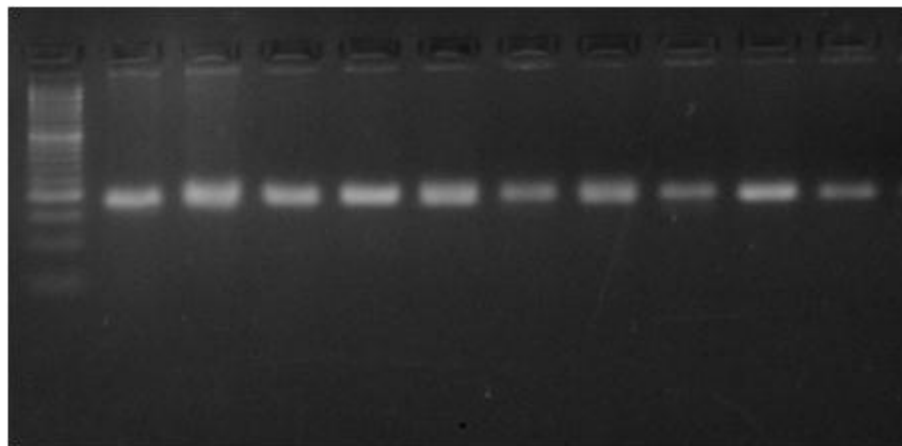
After 44 h, all the hygienic colonies had reached 100 per cent removal of the inoculated brood and among the non-hygienic colonies, it ranged between 92.05-96.81 per cent with a mean of 94.7 per cent. The non-hygienic colonies recorded 100 per cent removal of the inoculated brood cells after 52 h of inoculation (Table 39, Fig. 28 and 29).

A058



M 1NH 2NH 3NH 1H 2H 3H 4H 5H 6H 7H M

AT128



M 1H 2H 3H 4H 5H 6H 7H 1NH 2NH 3NH

Fig. 27 Gel electrophoresis profiles of PCR amplified DNA of wing clips of queens representing hygienic (1H-7H) and non hygienic colonies (1NH-3NH) with SSR markers A058 and AT128. Lane M 50bp DNA Marker; Lanes 1-10 DNA amplified of queens o colonies 1H-7H and 1NH-3NH.

Table 37. *Varroa destructor* inoculated brood removal at various time intervals after the inoculations during autumn 2016

Col. No.	Cells emptied							
	2h		4h		6h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	0.00±0.00 (1.00)	0	0.67±0.33 (1.28)	3.19	2.00±0.33 (1.73)	9.52	0.89±0.59 (1.34)	4.24
2H	0.33±0.33 (1.14)	1.57	0.33±0.33 (1.14)	1.57	1.00±0.00 (1.41)	4.76	0.56±0.22 (1.23)	2.67
3H	0.67±0.33 (1.28)	3.19	0.67±0.33 (1.28)	3.19	1.33±0.33 (1.52)	6.33	0.89±0.22 (1.36)	4.24
4H	0.00±0.00 (1.00)	0	0.33±0.33 (1.14)	1.57	1.00±0.00 (1.41)	4.76	0.44±0.29 (1.18)	2.10
5H	0±0.00 (1.00)	0	0.67±0.33 (1.28)	3.19	1.00±0.00 (1.41)	4.76	0.56±0.29 (1.23)	2.67
6H	0.67±0.33 (1.28)	3.19	1.00±0.00 (1.41)	4.76	1±0.00 (1.41)	4.76	0.89±0.11 (1.37)	4.24
7H	0.33±0.33 (1.14)	1.57	1.00±0.00 (1.41)	4.76	1±0.00 (1.41)	4.76	0.78±0.22 (1.32)	3.71
1NH	0±0.00 (1.00)	0	0.33±0.33 (1.14)	1.57	0.67±0.33 (1.28)	3.19	0.33±0.19 (1.14)	1.57
2NH	0±0.00 (1.00)	0	0±0.00 (1.00)	0	0.33±0.33 (1.14)	1.57	0.11±0.11 (1.05)	0.52
3NH	0±0.00 (1.00)	0	0±0.00 (1.00)	0	0.33±0.33 (1.14)	1.57	0.11±0.11 (1.05)	0.52
Mean	0.2 1.08	-	0.5 1.21	-	0.97 1.39	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony	(0.15)
Time	(0.08)
Colony x Time	NS

Table 38. *Varroa destructor* inoculated brood cells emptied recorded at various intervals after 20h of inoculation during autumn 2016

Col. No.	Cells emptied													
	20h		22h		24h		26h		28h		30h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	16.33±0.67 (4.16)	77.76	17.00±0.58 (4.24)	80.95	18.67±0.33 (4.43)	88.90	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	19.17±1.24 (4.48)	91.28
2H	18.00±0.58 (4.36)	85.71	19.33±0.88 (4.51)	92.05	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	20.22±0.74 (4.60)	96.28
3H	18.33±0.33 (4.40)	87.29	18.33±0.33 (4.40)	87.29	19.67±0.33 (4.55)	93.67	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	19.89±0.76 (4.57)	94.71
4H	16.00±0.00 (4.12)	76.19	16.33±0.33 (4.16)	77.76	18.00±0.00 (4.36)	85.71	19.33±0.33 (4.51)	92.05	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	18.61±1.27 (4.42)	88.62
5H	16.00±0.00 (4.12)	76.19	17.00±0.00 (3.46)	80.95	18.00±0.00 (4.12)	85.71	19.00±0.00 (4.36)	90.48	20.00±0.00 (4.43)	95.24	20.00±0.00 (4.43)	95.24	18.33±0.94 (4.39)	87.28
6H	18.67±0.33 (4.43)	88.90	20.33±0.67 (4.62)	96.81	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	20.50±0.54 (4.64)	97.62
7H	18.33±0.33 (4.40)	87.29	18.67±0.33 (4.43)	88.90	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	20.17±0.75 (4.60)	96.05
1NH	9.00±0.00 (3.16)	42.86	9.00±0.58 (3.16)	42.86	11.33±0.33 (3.51)	53.95	11.00±0.58 (3.46)	52.38	14.33±0.33 (3.92)	68.24	15.00±0.00 (4.00)	71.83	11.61±1.48 (3.53)	55.28
2NH	10.00±0.00 (3.32)	47.62	12.00±0.00 (3.61)	57.14	13.00±0.00 (3.74)	61.90	15.67±0.33 (4.08)	74.62	17.67±0.33 (4.32)	84.14	17.67±0.33 (4.32)	84.14	14.33±1.83 (3.90)	68.24
3NH	12.33±0.33 (3.65)	58.71	12.33±0.33 (3.65)	58.71	14.67±0.33 (3.96)	69.86	15.33±0.67 (4.04)	73	18.00±0.00 (4.36)	85.71	18.00±0.00 (4.36)	85.71	15.11±1.47 (4.00)	71.95
Mean	15.30 (4.01)	-	16.03 (4.10)	-	17.63 (4.30)	-	18.53 (4.40)	-	19.60 (4.53)	-	19.67 (4.54)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony (0.15)

Time (0.08)

Colony x Time NS

Table 39. *Varroa destructor* inoculated brood cells emptied recorded at various intervals after 44h of inoculation during autumn 2016

Col. No.	Cells emptied											
	44h		46h		48h		50h		52h		Mean	
	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
1H	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100
2H	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100
3H	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100
4H	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100
5H	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100
6H	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100
7H	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100
1NH	19.33±0.33 (4.51)	92.05	19.33±0.33 (4.51)	92.05	20.00±0.00 (4.58)	95.24	20.00±0.00 (4.58)	95.24	21.00±0.00 (4.69)	100	19.93±0.43 (4.54)	94.90
2NH	20.33±0.67 (4.62)	96.81	20.33±0.67 (4.62)	96.81	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	20.73±0.20 (4.66)	98.71
3NH	20.00±0.00 (4.58)	95.24	20.00±0.00 (4.58)	95.24	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	21.00±0.00 (4.69)	100	20.60±0.30 (4.65)	98.09
Mean	20.67 (4.65)	-	20.67 (4.65)	-	20.90 (4.68)	-	20.90 (4.68)	-	21.00 (4.69)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony	(0.15)
Time	(0.08)
Colony x Time	NS

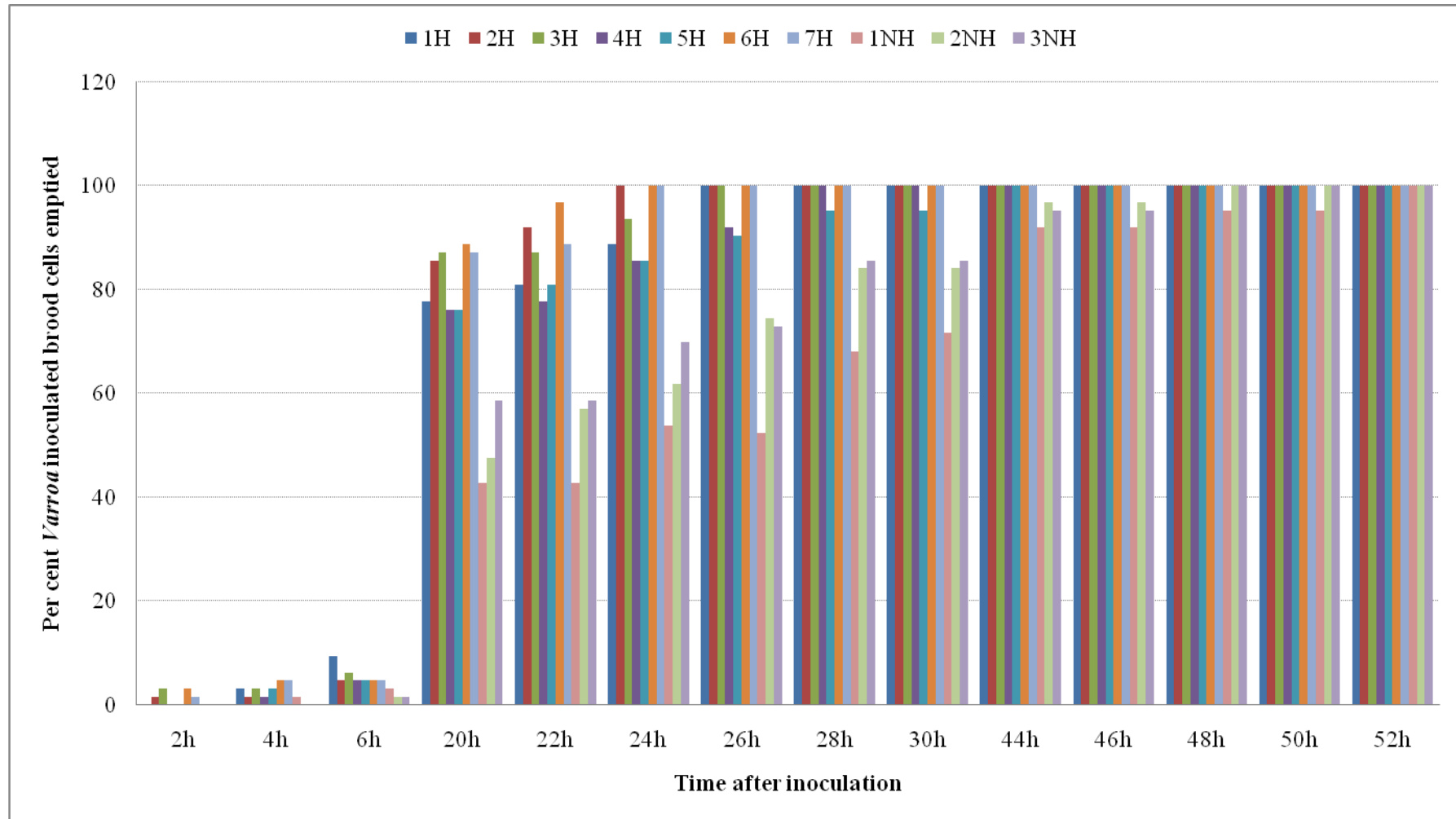


Fig. 28 Percentage of brood cells emptied in various test colonies by various time intervals after *Varroa* inoculation during autumn 2016

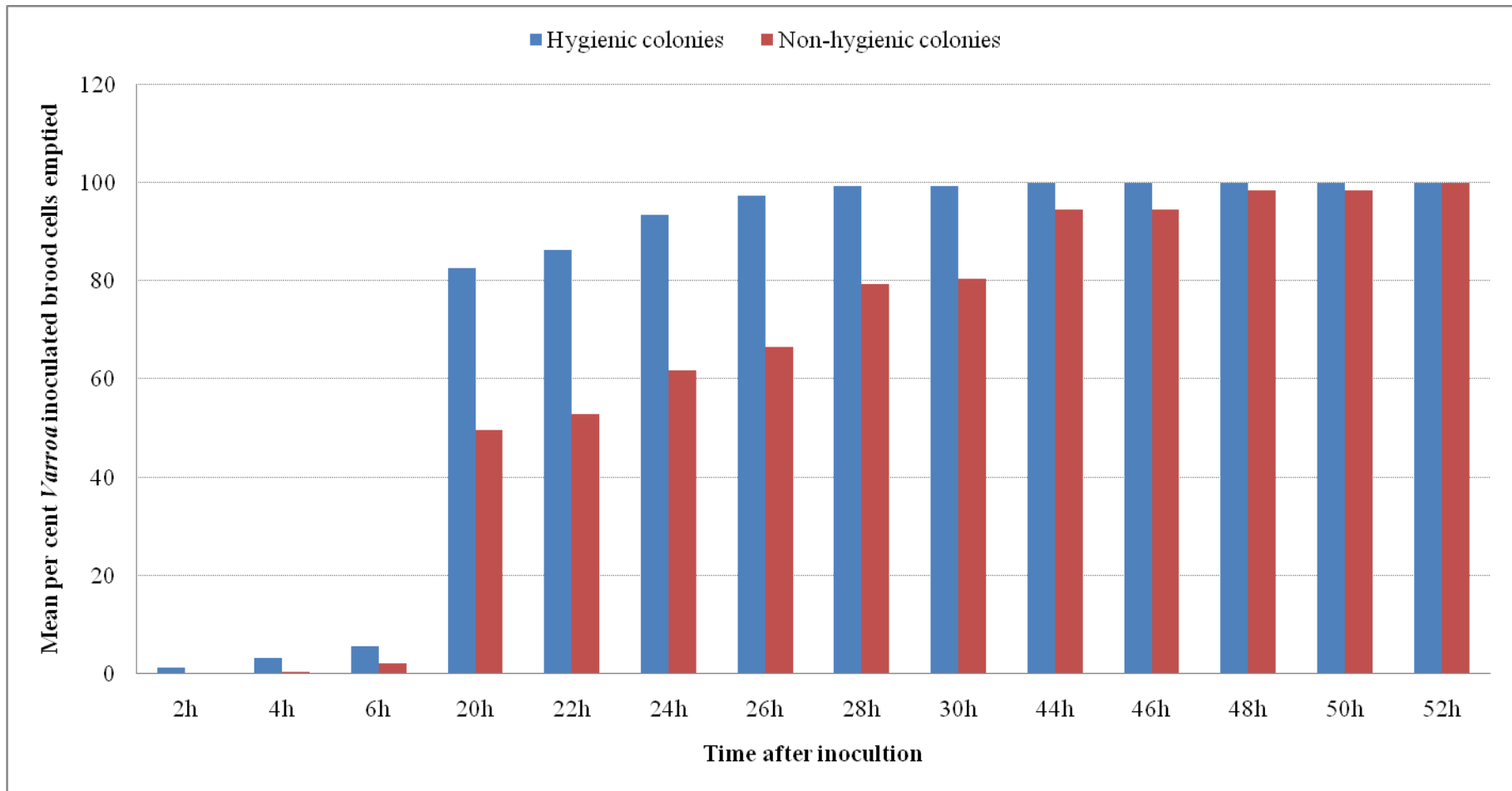


Fig. 29 Mean percentage of brood cells emptied in hygienic and non-hygienic colonies at various time intervals after *Varroa* inoculation during autumn 2016

4.8.1.2 *V. destructor* inoculated brood cells only uncapped after various time intervals

For the seven hygienic colonies, the mean *Varroa* inoculated brood cells only uncapped was 14.52 (range of 9.52-22.24%), 17.90 (range: 11.10-22.24%) and 18.59 per cent (range: 11.10-23.81%) after 2, 4 and 6 h of inoculation, respectively, while for the non-hygienic colonies, the corresponding mean values were 4.76 (range: 1.57-7.95%), 6.35 (range: 3.19-9.52%) and 6.35 per cent (range: 3.19-11.10%) (Table 40, Fig. 30 and 31).

After 20 h (Table 41 and 42, Fig. 30 and 31), 3.62 per cent (1.57-7.95%) mite inoculated brood cells were only uncapped in the hygienic colonies and 1.59 per cent (0.00-4.765) in non-hygienic colonies. In the hygienic colonies, the mean of 4.3, 3.17, 1.36, 1.81, 0.68 and 0.22 per cent of mite inoculated brood cells were only uncapped, while in the non-hygienic colonies, it was 2.11, 3.7, 4.76, 1.06, 2.65 and 2.11 per cent after 22, 24, 26, 28, 30 and 44 h, respectively. After 46 h, the mean percentage of only uncapped brood cells in hygienic colonies was 0.22, while in non-hygienic colonies; it was 2.11 and continued to be so even till 50 h of inoculation.

4.8.1.3 *V. destructor* inoculated brood cells remaining capped

The mean percentage of brood cells remaining capped after 2, 4 and 6 h of inoculation was 84.12 (range: 74.62-87.28%), 78.68 (range: 73.00-84.14%) and 75.97 per cent (range: 71.43-84.14%) in hygienic colonies, and 95.24 (range: 92.05-98.43%), 93.13 (range: 90.48-95.24) and 92.08 per cent (range: 88.90-93.67%) in the non-hygienic colonies, respectively (Table 43, Fig. 32 and 33).

At 20 h after the inoculation a mean of 13.14 (range: 6.33-23.81%) and 48.68 per cent (range: 41.28-57.14%) inoculated brood cells remained capped in the hygienic and non-hygienic colonies, respectively (Table 44, Fig. 32 and 33). After 24 h, in the hygienic colonies mean inoculated brood cells remaining capped was 2.72 per cent and in the non-hygienic colonies, it was 34.39 per cent. After 28 h, when 0.00 per cent of brood cells were left uncapped in hygienic colonies, an average of 19.57 per cent mite inoculated brood cells were recorded as capped in the non-hygienic colonies.

Though the hygienic colonies had achieved cent per cent brood cells as uncapped, the data was continued to be recorded for the non-hygienic colonies the next day after 44 h of inoculation i.e. till no brood cells remained capped (Table 45, Fig. 32 and 33).

4.8.2 Time interval to attain 100 per cent uncapping and cleaning of inoculated brood c

To achieve 100 per cent uncapping and cleaning of cells, the hygienic colonies on an average took 28 h whereas non-hygienic colonies took 50.67 h to achieve the same in autumn season.

Table 40. *Varroa destructor* inoculated brood cells only uncapped at various time intervals after inoculations during autumn 2016

Col. No.	Cells only uncapped							
	2 h		4 h		6 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	3.00±0.00 (2.00)	14.29	4.33±0.33 (2.31)	20.62	4.00±0.00 (2.24)	19.05	3.78±0.40 (2.18) ^{ab}	18.9
2H	3.67±0.33 (2.16)	17.48	4.33±0.33 (2.31)	20.62	5.00±0.00 (2.45)	23.81	4.33±0.38 (2.30) ^{ac}	20.62
3H	4.67±0.33 (2.38)	22.24	4.67±0.33 (2.38)	22.24	4.67±0.33 (2.38)	22.24	4.67±0.00 (2.38) ^c	22.24
4H	2.67±0.33 (1.91)	12.71	4.33±0.67 (2.30)	20.62	4.33±0.33 (2.31)	20.62	3.78±0.55 (2.17) ^a	18
5H	2.67±0.33 (1.91)	12.71	3.33±0.33 (2.08)	15.86	4.00±0.00 (2.44)	19.05	3.33±0.38 (2.08) ^b	15.86
6H	2.00±0.58 (1.72)	9.52	2.33±0.33 (1.82)	11.10	2.33±0.33 (1.82)	11.10	2.22±0.11 (1.79) ^d	10.57
7H	2.67±0.33 (1.91)	12.71	3.00±0.00 (2.00)	14.29	3.00±0.00 (2.00)	14.29	2.89±0.11 (1.97) ^e	13.76
1NH	0.33±0.33 (1.14)	1.57	0.67±0.33 (1.28)	3.19	0.67±0.67 (1.24)	3.19	0.56±0.11 (1.22) ^f	2.67
2NH	1.00±0.00 (1.41)	4.76	1.33±0.33 (1.52)	6.33	1.00±0.00 (1.41)	4.76	1.11±0.11 (1.45) ^g	5.59
3NH	1.67±0.33 (1.63)	7.95	2.00±0.00 (1.73)	9.52	2.33±0.33 (1.82)	11.10	2.00±0.19 (1.73) ^d	9.52
Mean	2.43 1.82	-	3.037 1.97	-	3.13 1.99	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony	(0.15)
Time	(0.08)
Colony x Time	NS

Table 41. *Varroa destructor* inoculated brood cells only uncapped recorded at various time intervals after 20h of inoculations during autumn 2016

Col. No.	Cells only uncapped													
	20h		22h		24h		26h		28h		30h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	0.33±0.33 (1.14)	1.57	0.33±0.33 (1.14)	1.57	2.33±0.33 (1.14)	11.09	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.16±0.53 (1.18)	2.38
2H	1.67±0.33 (1.63)	7.95	1.33±0.67 (1.49)	6.33	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.50±0.45 (1.19)	2.38
3H	1.33±0.33 (1.52)	6.33	2.67±0.33 (1.91)	12.71	1.33±0.33 (1.52)	6.33	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.89±0.63 (1.33)	4.24
4H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	1.00±0.00 (1.41)	4.76	1.00±0.00 (1.41)	4.76	1.67±0.33 (1.63)	7.95	0.00±0.00 (1.00)	0.00	0.61±0.41 (1.24)	2.90
5H	0.00±0.00 (1.00)	0.00	0.67±0.33 (1.28)	3.19	1.00±0.00 (1.41)	4.76	1.00±0.00 (1.41)	4.76	1.00±0.00 (1.41)	4.76	1.00±0.00 (1.41)	4.76	0.78±0.23 (1.32)	3.71
6H	1.00±0.00 (1.41)	4.76	0.33±0.33 (1.14)	1.57	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.22±0.23 (1.09)	1.05
7H	1.00±0.00 (1.41)	4.76	1.00±0.58 (1.38)	4.76	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.33±0.24 (1.13)	1.57
1NH	0.00±0.00 (1.00)	0.00	1.33±0.33 (1.52)	6.33	1.00±0.00 (1.41)	4.76	2.33±0.67 (1.80)	11.09	0.67±0.33 (1.28)	3.19	1.00±0.00 (1.41)	4.76	1.06±0.45 (1.40)	5.05
2NH	1.00±0.00 (1.41)	4.76	0.00±0.00 (1.00)	0.00	0.33±0.33 (1.14)	1.57	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.22±0.23 (1.09)	1.05
3NH	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	1.00±0.00 (1.41)	4.76	0.67±0.33 (1.28)	3.19	0.00±0.00 (1.00)	0.00	0.67±0.33 (1.28)	3.19	0.39±0.26 (1.16)	1.86
Mean	0.63 (1.25)	-	0.77 (1.29)	-	0.80 (1.31)	-	0.50 (1.19)	-	0.33 (1.13)	-	0.27 (1.11)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony	(0.15)
Time	(0.08)
Colony x Time	NS

Table 42. *Varroa destructor* inoculated brood cells only uncapped recorded at various time intervals after 44 h of inoculation during autumn 2016

Col. No.	Cells only uncapped											
	44 h		46 h		48 h		50 h		52 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Percent
1H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
2H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
3H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
4H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
5H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
6H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
7H	0.33±0.33 (1.14)	1.57	0.00±0.00 (1.00)	1.57	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.06±0.08 (1.02)	0.28
1NH	1.00±0.00 (1.41)	4.76	1.00±0.00 (1.41)	4.76	1.00±0.00 (1.41)	4.76	1.00±0.00 (1.41)	4.76	0.00±0.00 (1.00)	0.00	0.80±0.30 (1.33)	3.81
2NH	0.33±0.33 (1.14)	1.57	0.33±0.33 (1.14)	1.57	0.33±0.00 (1.14)	1.57	0.33±0.00 (1.14)	1.57	0.00±0.00 (1.00)	0.00	0.26±0.10 (1.11)	1.24
3NH	0.00±0.00 (1.41)	0.00	0.00±0.00 (1.41)	0.00	0.00±0.00 (1.41)	0.00	0.00±0.00 (1.41)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.14)	0.00
Mean	0.17 (1.11)	-	0.13 (1.10)	-	0.10 (1.04)	-	0.10 (1.04)	-	0.00 (1.00)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony (0.15)

Time (0.08)

Colony x Time NS

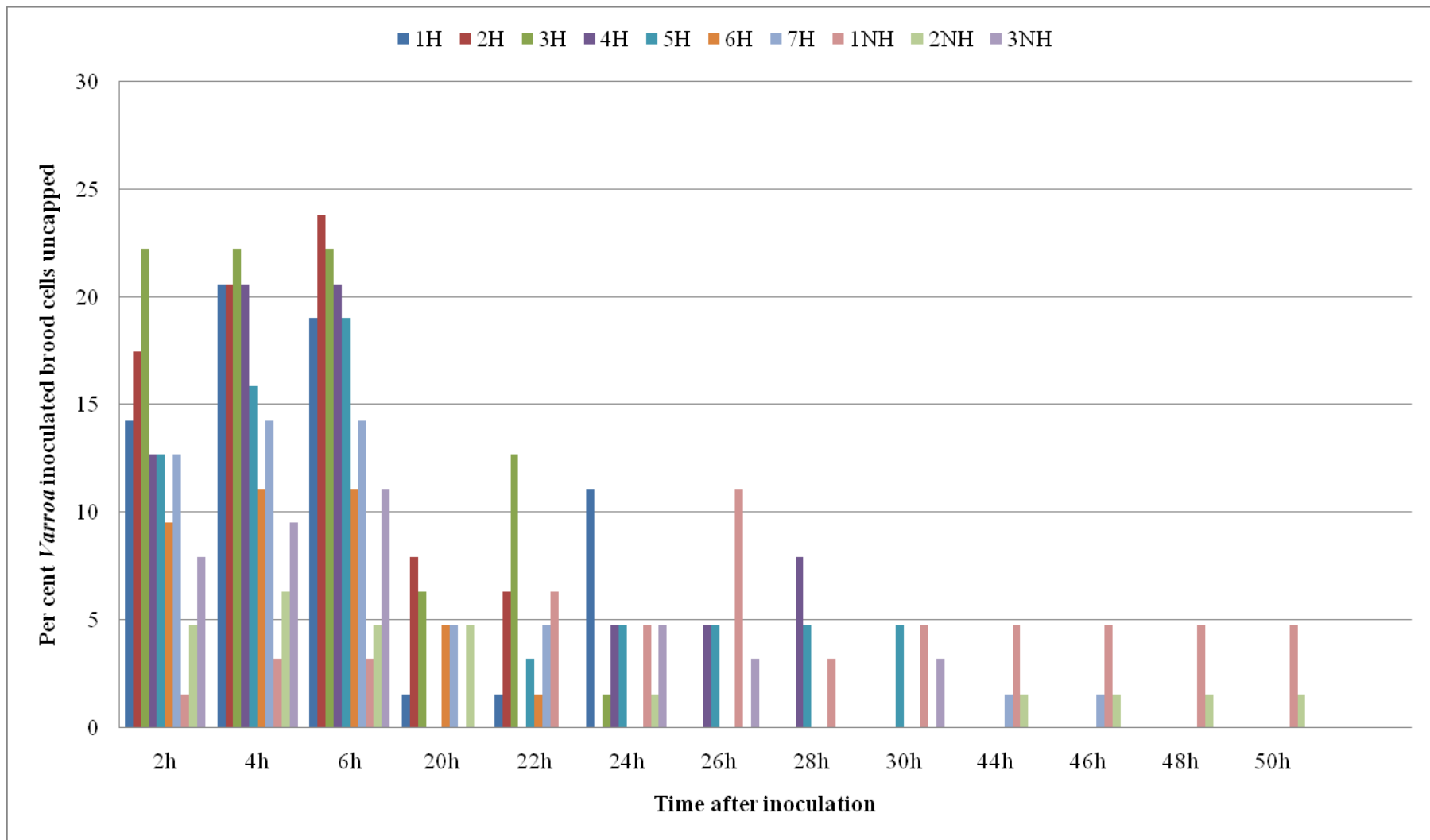


Fig. 30 Percentage of brood cells only uncapped in various test colonies at various time intervals after *Varroa* inoculation during autumn 2016

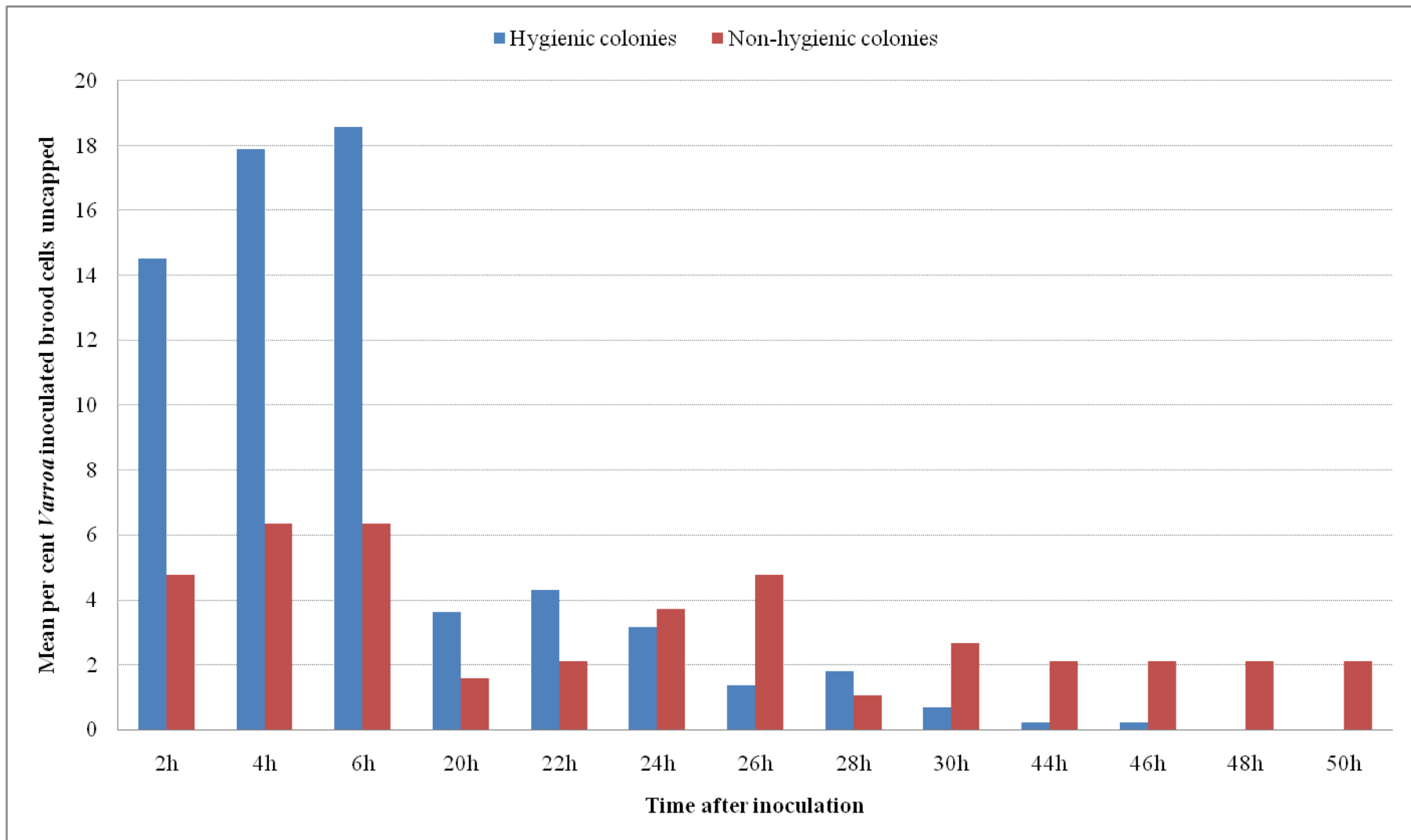


Fig. 31 Mean percentage of brood cells only uncapped in hygienic and non-hygienic colonies at various time intervals after *Varroa* inoculation during autumn 2016

Table 43. *Varroa destructor* inoculated brood cells remaining capped at various time intervals after the inoculations during autumn 2016

Col. No.	Cells remaining capped							
	2 h		4 h		6 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	18.00±0.00 (4.36)	85.71	16.00±0.00 (4.12)	76.19	15.00±0.00 (4.00)	71.43	16.33±0.88 (4.16)	77.76
2H	17.00±0.00 (4.24)	80.95	16.33±0.33 (4.16)	77.76	15.33±0.33 (4.04)	73	16.22±0.49 (4.15)	77.24
3H	15.67±0.33 (4.08)	74.62	15.33±0.33 (4.04)	73	15.00±0.00 (4.00)	71.43	15.33±0.19 (4.04)	73
4H	18.33±0.33 (4.40)	87.28	16.33±0.33 (4.16)	77.76	15.67±0.33 (4.08)	74.62	16.78±0.80 (4.21)	79.90
5H	18.33±0.33 (4.40)	87.28	17.00±0.00 (4.24)	80.95	16.00±0.00 (4.12)	76.19	17.11±0.67 (4.25)	81.48
6H	18.33±0.33 (4.40)	87.28	17.67±0.33 (4.32)	84.14	17.67±0.33 (4.32)	84.14	17.89±0.22 (4.35)	85.19
7H	18.00±0.00 (4.36)	85.71	17.00±0.00 (4.24)	80.95	17.00±0.00 (4.24)	80.95	17.33±0.33 (4.28)	82.52
1NH	20.67±0.33 (4.65)	98.43	20.00±0.00 (4.58)	95.24	19.67±0.33 (4.55)	93.67	20.11±0.29 (4.59)	95.28
2NH	20.00±0.00 (4.58)	95.24	19.67±0.33 (4.55)	93.67	19.67±0.33 (4.55)	93.67	19.78±0.11 (4.56)	94.19
3NH	19.33±0.33 (4.51)	92.05	19.00±0.00 (4.47)	90.48	18.67±0.33 (4.43)	88.90	19.00±0.19 (4.47)	90.48
Mean	18.37 (4.40)	-	17.43 (4.29)	-	16.97 (4.23)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony	(0.15)
Time	(0.08)
Colony x Time	NS

Table 44. *Varroa destructor* inoculated brood remaining capped recorded at various time intervals after 20 h of inoculation during autumn 2016

Col. No.	Cells remaining capped													
	20 h		22 h		24 h		26 h		28 h		30 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	4.33±0.33 (2.31)	20.62	3.67±0.33 (2.16)	17.48	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	1.33±1.20 (1.41)	6.33
2H	1.33±0.33 (1.52)	6.33	0.33±0.33 (1.14)	1.57	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.28±0.31 (1.11)	1.33
3H	1.33±0.33 (1.52)	6.33	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.22±0.31 (1.09)	1.05
4H	5.00±0.00 (3.16)	23.81	4.67±0.33 (2.38)	22.24	2.00±0.00 (1.73)	9.52	0.67±0.33 (1.28)	3.19	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	2.06±1.31 (1.64)	9.81
5H	4.33±0.33 (2.31)	20.62	3.33±0.33 (2.08)	15.86	2.00±0.00 (1.73)	9.52	1.00±0.00 (1.41)	4.76	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	1.78±1.03 (1.59)	8.48
6H	1.33±0.33 (1.52)	6.33	0.33±0.33 (1.14)	1.57	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.28±0.31 (1.11)	1.33
7H	1.67±0.33 (1.63)	7.95	1.33±0.33 (1.52)	6.33	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.50±0.45 (1.19)	2.38
1NH	12.00±0.00 (3.61)	57.14	10.67±0.33 (3.41)	50.81	8.67±0.33 (3.11)	41.28	7.67±0.33 (2.94)	36.52	6.00±0.00 (2.65)	28.57	5.00±0.00 (2.45)	23.81	8.33±1.55 (3.03)	39.67
2NH	10.00±0.00 (3.32)	47.62	9.00±0.00 (3.16)	42.86	7.67±0.33 (2.94)	36.52	5.33±0.33 (2.51)	25.38	3.33±0.33 (2.08)	15.86	3.33±0.33 (2.08)	15.86	6.44±1.66 (2.68)	30.67
3NH	8.67±0.33 (3.11)	41.28	8.67±0.33 (3.11)	41.28	5.33±0.33 (2.51)	25.38	5.00±0.58 (2.44)	23.81	3.00±0.00 (2.00)	14.28	2.33±0.33 (1.82)	11.09	5.00±1.56 (2.50)	23.81
Mean	6.07 (2.33)	-	5.07 (2.11)	-	2.87 (1.70)	-	2.23 (1.56)		1.50 (1.37)	-	1.33 (1.33)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony (0.15)

Time (0.08)

Colony x Time NS

Table 45. *Varroa destructor* inoculated brood remaining capped recorded at various time intervals after 44 h of inoculation during autumn 2016

Col. No.	Cells remaining capped											
	44 h		46 h		48 h		50 h		52 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Percent
1H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
2H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
3H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
4H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
5H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
6H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
7H	0.00±0.00 (1.14)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.14)	0.00	0.00±0.00 (1.02)	0.00
1NH	0.67±0.33 (1.28)	3.19	0.67±0.33 (1.28)	3.19	0.67±0.00 (1.28)	3.19	0.67±0.00 (1.28)	3.19	0.00±0.00 (1.00)	0.00	0.54±0.00 (1.19)	2.57
2NH	0.33±0.33 (1.14)	1.57	0.33±0.33 (1.14)	1.57	0.33±0.00 (1.14)	1.57	0.33±0.00 (1.14)	1.57	0.00±0.00 (1.00)	0.00	0.26±0.00 (1.11)	1.24
3NH	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
Mean	0.10 (1.06)	-	0.10 (1.04)	-	0.10 (1.06)	-	0.10 (1.06)	-	0.00 (1.00)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony (0.15)
Time (0.08)
Colony x Time NS

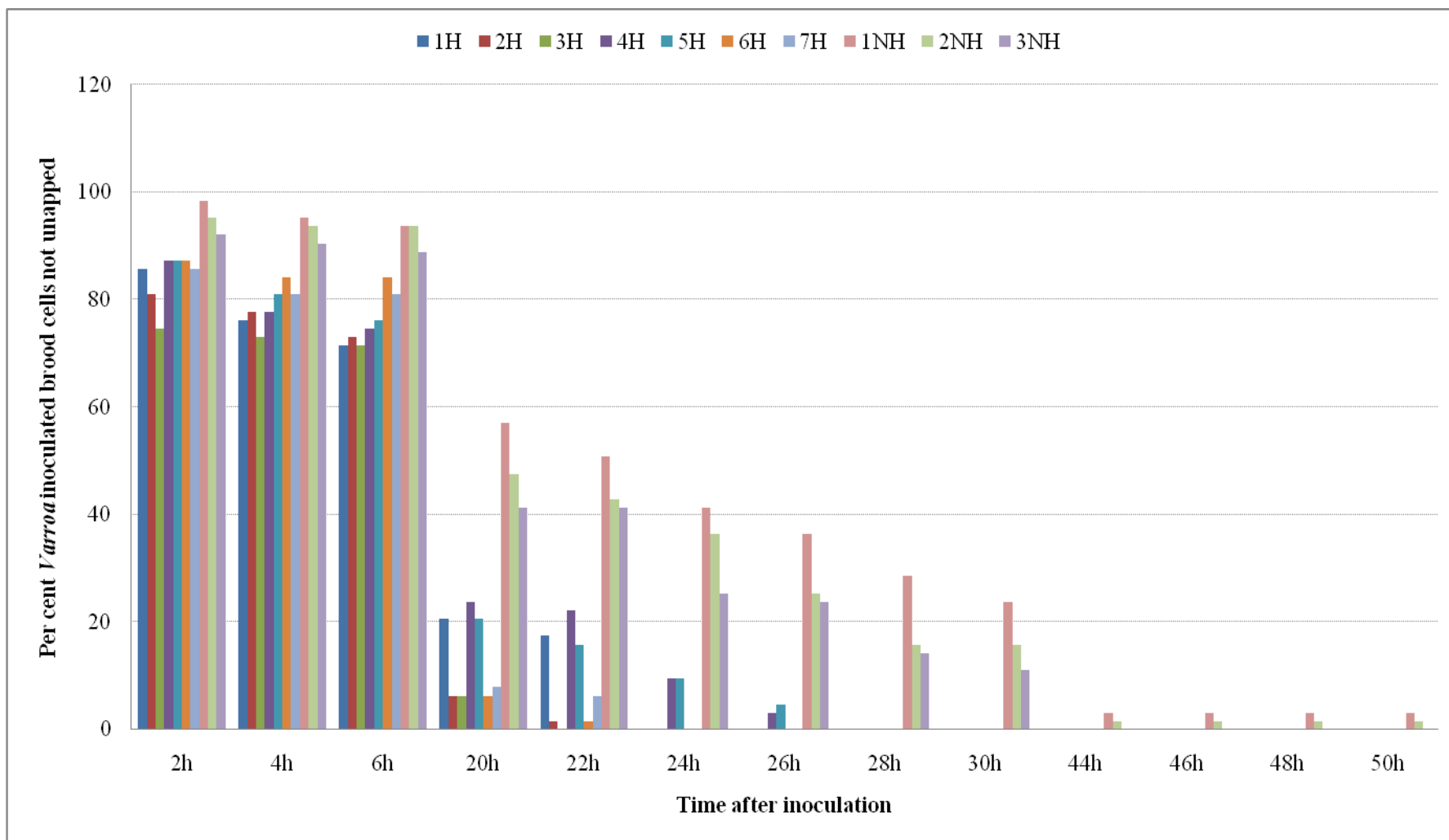


Fig. 32 Percentage of brood cells remaining capped in various test colonies at various time intervals after *Varroa* inoculation during autumn 2016

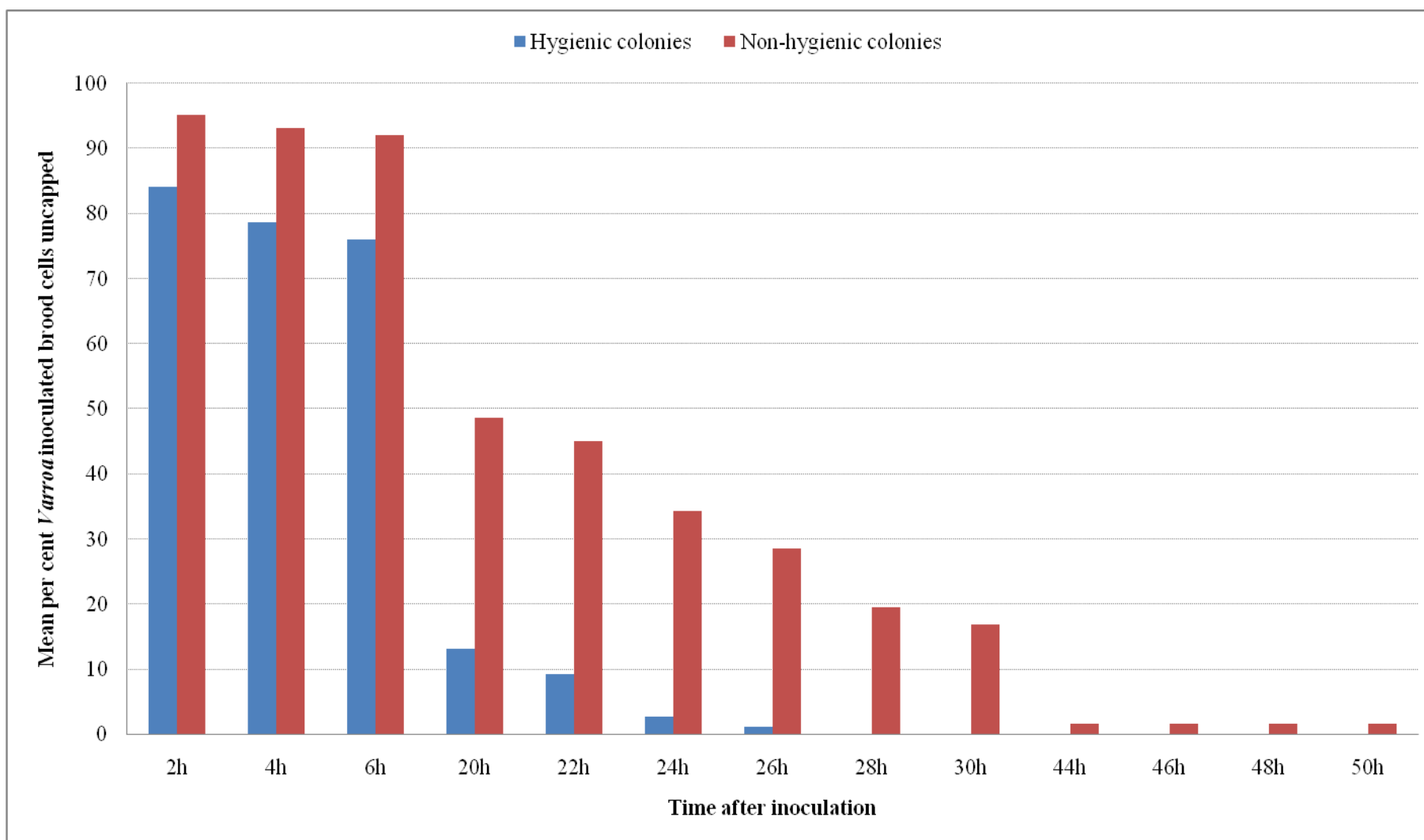


Fig. 33 Mean percentage of brood cells remaining capped in hygienic and non-hygienic colonies at various time intervals after *Varroa* inoculation during autumn 2016

4.8.3 Validation during spring season, 2017

4.8.3.1 *V. destructor* inoculated brood removal

In the seven hygienic colonies, the mean percentage of *Varroa* mite inoculated brood cells emptied after 2, 4 and 6 h was 3.62 (range: 0.00-7.95%) ; 6.57 (range: 4.76-9.52%) and 7.25 per cent (range:6.33-9.52%), respectively. In the non-hygienic colonies, the mean was 0 per cent; 1.57 and 2.11 per cent (range: 1.57- 3.19%) after 2, 4 and 6 h, respectively (Table 46, Fig. 34 and 35).

In the hygienic colonies, the percentage of inoculated brood cells emptied ranged between 82.52- 95.24 per cent after 20 h of inoculation and in the non- hygienic colonies at this hour, the brood cells emptied were in the range of 71.43-76.19 per cent. After 24 h of inoculated brood cells, in the seven selected hygienic colonies, a mean of 95.24 per cent of brood cells removal was recorded (range: 88.90-100%), while in the three non-hygienic colonies, the mean brood removal was only 77.24 per cent (range: 76.19-77.76%).

After 30 h of inoculation, six hygienic colonies had achieved 100 per cent removal of inoculated brood cells while in non-hygienic colonies it was in the range of 80.95-85.71 per cent (Table 47, and Fig. 34 and 35).

After 44 h all the hygienic colonies had reached 100 per cent removal of inoculated brood cells, and in non-hygienic colonies, it was in the range of 92.05-98.43 per cent with a mean of 95.24 per cent. The non-hygienic colonies recorded 100 per cent removal of inoculated brood cells after 52 h of inoculation (Table 48, Fig. 34 and 35).

4.8.3.2 *V. destructor* inoculated brood cells only uncapped

In the seven hygienic colonies, the mean of *Varroa* inoculated brood cells only uncapped was 17.23, 19.96 and 19.73 per cent after 2, 4 and 6 h of inoculation, respectively, and the (range:11.09-23.81%) was same during all the time durations. In the non-hygienic colonies, the mean was 12.16 (range: 9.52-15.86%); 12.17 (range: 3.19-9.52%) and 11.12 per cent (range: 7.95-12.71%), after 2, 4 and 6 h of inoculations, respectively (Table 49, Fig. 36 and 37).

After 20 h (Table 50 and 51, Fig. 36 and 37), the mean of 5.22 per cent mite inoculated brood cells were only uncapped in the hygienic colonies and 7.93 per cent (0.00-4.765) in non-hygienic colonies. In the hygienic colonies, the mean of 5.21, 2.49, 0.9, 0.68,0.68, 0.22 and 0.00 per cent of mite inoculated brood cells were found only uncapped, while in the non-hygienic colonies, it was 10.04, 11.07, 9.52, 9.52, 10.04 and 1.59 per cent after 22 , 24 , 26 , 28 h, 30, 44 and 46 h, respectively.

Table 46. *Varroa destructor* inoculated brood removed at various time intervals after the inoculations during spring 2017

Col. No.	Cells emptied							
	2 h		4 h		6 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	0.00±0.00 (1.00)	0.00	1.00±0.00 (1.41)	4.76	1.33±0.33 (1.52)	6.33	0.78±0.40 (1.31)	3.71
2H	0.33±0.33 (1.14)	1.57	1.00±0.00 (1.41)	4.76	1.33±0.33 (1.52)	6.33	0.89±0.29 (1.36)	4.24
3H	1.67±0.33 (1.63)	7.95	2.00±0.00 (1.73)	9.52	2.00±0.00 (1.73)	9.52	1.87±0.11 (1.70)	8.90
4H	0.33±0.33 (1.14)	1.57	1.33±0.33 (1.52)	6.33	1.33±0.33 (1.52)	6.33	1.00±0.33 (1.39)	4.76
5H	0.33±0.33 (1.14)	1.57	1.00±0.00 (1.41)	4.76	1.33±0.33 (1.52)	6.33	0.80±0.29 (1.36)	3.81
6H	1.00±0.00 (1.41)	4.76	1.67±0.33 (1.63)	7.95	1.67±0.33 (1.63)	7.95	1.44±0.22 (1.56)	0.07
7H	1.67±0.33 (1.63)	7.95	1.67±0.33 (1.63)	7.95	1.67±0.33 (1.63)	7.95	1.67±0.00 (1.63)	7.95
1NH	0.00±0.00 (1.00)	0.00	0.33±0.33 (1.14)	1.57	0.67±0.33 (1.52)	3.19	0.33±0.19 (1.14)	1.57
2NH	0.00±0.00 (1.00)	0.00	0.33±0.33 (1.14)	1.57	0.33±0.33 (1.14)	1.57	0.22±0.11 (1.09)	1.05
3NH	0.00±0.00 (1.00)	0.00	0.33±0.33 (1.14)	1.57	0.33±0.33 (1.14)	1.57	0.22±0.11 (1.09)	1.05
Mean	0.53± (1.21)	-	1.07± (1.42)	-	1.20± (1.40)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony (0.15)

Time (0.08)

Colony x Time NS

Table 47. *Varroa destructor* inoculated brood cells emptied recorded at various time intervals after 20 h of inoculations during spring 2017

Col. No.	Cells emptied													
	20 h		22 h		24 h		26 h		28 h		30 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	18.00±0.00 (4.36)	85.71	18.33±0.33 (4.40)	87.28	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	20.05±0.85 (4.59)	95.48
2H	18.33±0.33 (4.40)	87.28	18.33±0.33 (4.40)	87.28	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	20.11±0.80 (4.59)	95.76
3H	19.00±0.00 (4.47)	90.48	19.00±0.00 (4.47)	90.48	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	20.33±0.60 (4.62)	96.81
4H	17.33±0.33 (4.28)	82.52	17.67±0.33 (4.32)	84.14	18.67±0.33 (4.42)	88.90	19.67±0.33 (4.54)	93.67	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	19.22±0.92 (4.49)	91.52
5H	19.00±0.00 (4.47)	90.48	19.00±0.00 (4.47)	90.48	19.67±0.33 (4.55)	93.67	19.67±0.33 (4.55)	93.67	20.00±0.00 (4.58)	95.24	20.00±0.00 (4.58)	95.24	19.56±0.33 (4.50)	93.14
6H	19.33±0.33 (4.51)	92.05	20.33±0.33 (4.62)	96.81	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	20.61±0.39 (4.65)	98.14
7H	20.00±0.58 (4.58)	95.24	20.00±0.58 (4.58)	95.24	20.67±0.33 (4.65)	98.43	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	20.50±0.28 (4.64)	97.62
1NH	15.33±0.33 (4.04)	73.00	15.33±0.33 (4.04)	73.00	16.00±0.00 (4.12)	76.19	17.00±0.00 (4.24)	80.95	17.33±0.33 (4.28)	82.52	17.33±0.33 (4.28)	82.52	16.39±0.55 (4.17)	78.05
2NH	15.00±0.00 (4.00)	71.43	16.00±0.00 (4.12)	76.19	16.33±0.33 (4.16)	77.76	16.33±0.33 (4.16)	77.76	16.33±0.33 (4.16)	77.76	17.00±0.33 (4.24)	80.95	16.70±0.38 (4.14)	79.52
3NH	16.00±0.58 (4.12)	76.19	16.33±0.33 (4.16)	77.76	16.33±0.33 (4.16)	77.76	17.00±0.00 (4.24)	80.95	18.00±0.00 (4.36)	85.71	18.00±0.00 (4.36)	85.71	16.94±0.51 (4.23)	80.67
Mean	17.33± (4.28)	-	17.33± (4.28)	-	18.87± (4.45)	-	19.30± (4.50)	-	19.63± (4.54)	-	19.70± (4.55)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony (0.15)

Time (0.08)

Colony x Time NS

Table 48. Number of *Varroa destructor* inoculated brood cells emptied recorded at various time intervals after 44 h of inoculation during spring season 2017

Col. No.	Cells emptied							
	44 h		46 h		48 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00
2H	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00
3H	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00
4H	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00
5H	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00
6H	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00
7H	20.67±0.33 (4.65)	98.43	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	20.89±0.08 (4.68)	99.48
1NH	19.33±0.33 (4.51)	92.05	21.00±0.00 (4.69)	100.00	21.00±0.00 (4.69)	100.00	20.72±0.39 (4.66)	98.67
2NH	20.67±0.33 (4.65)	98.43	20.67±0.33 (4.65)	98.43	21.00±0.00 (4.69)	100.00	20.44±0.16 (4.64)	97.35
3NH	20.00±0.00 (4.58)	95.24	20.00±0.00 (4.58)	95.24	21.00±0.00 (4.69)	100.00	20.33±0.30 (4.62)	96.82
Mean	20.67 (4.65)	-	20.87 (4.68)	-	21.00±0.00 (4.69)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony	(0.15)
Time	(0.08)
Colony x Time	NS

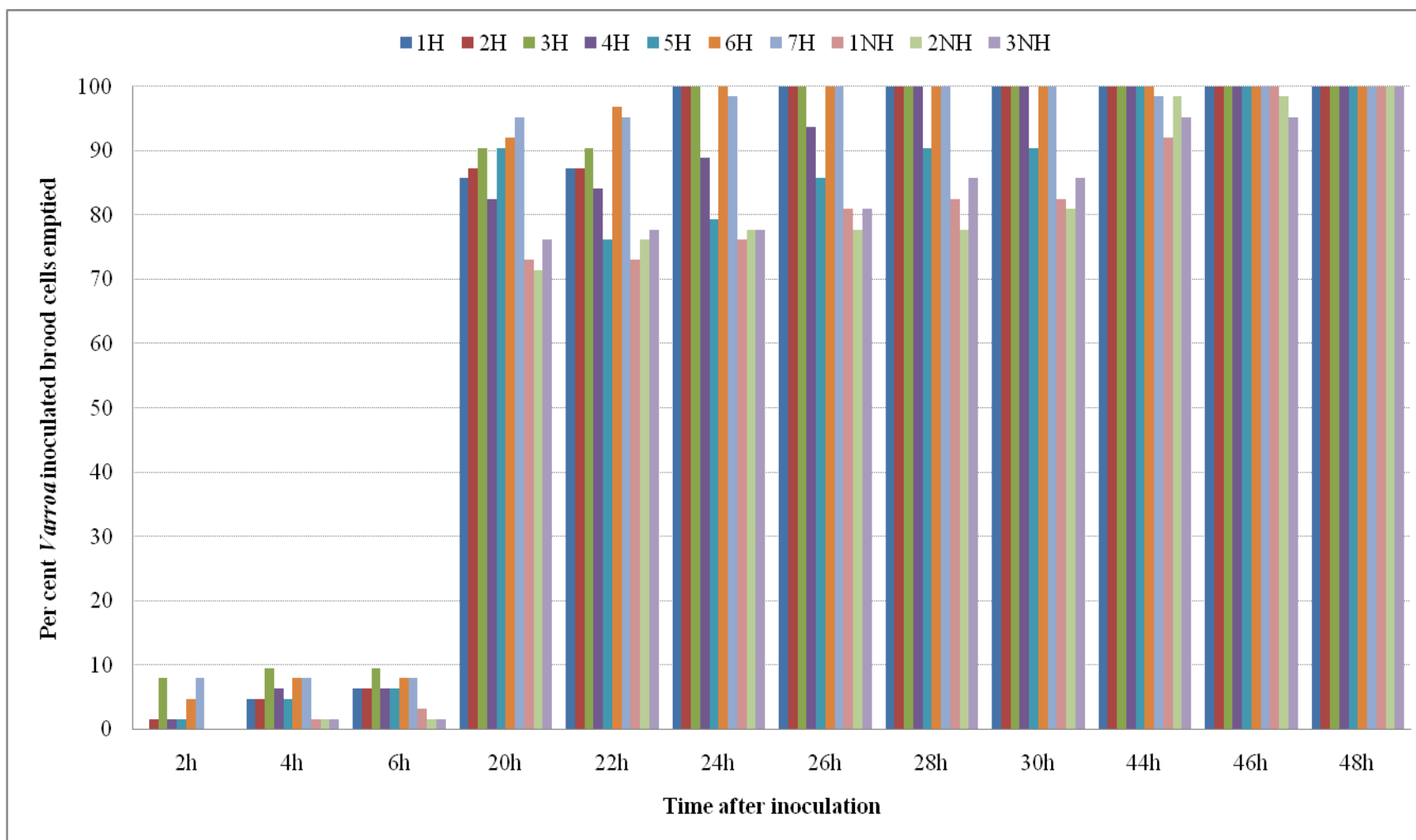


Fig. 34 Percentage of brood cells emptied in various test colonies by various time intervals after *Varroa* inoculation during spring 2017

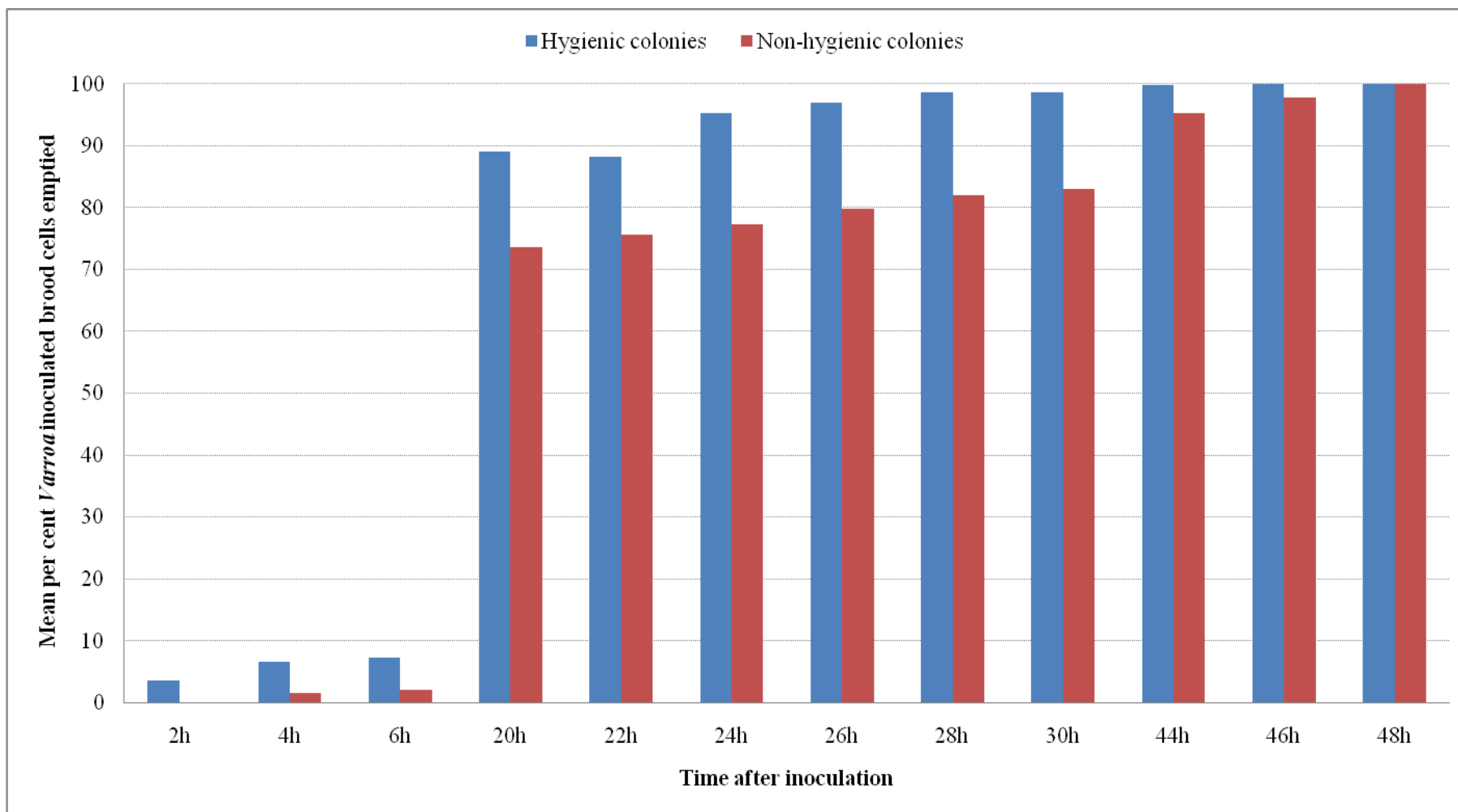


Fig. 35 Mean percentage of brood cells emptied in hygienic and non-hygienic colonies at various time intervals after *Varroa* inoculations during spring 2017

Table 49. *Varroa destructor* inoculated brood cells only uncapped at various time intervals after the inoculations during spring 2017

Col. No.	Cells only Uncapped							
	2 h		4 h		6 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	5.00±0.00 (2.45)	23.81	5.00±0.00 (2.45)	23.81	4.67±0.33 (2.38)	22.24	4.89±0.11 (2.42)	23.28
2H	4.33±0.33 (2.31)	20.62	4.67±0.33 (2.38)	22.24	4.67±0.33 (2.38)	22.24	4.56±0.11 (2.35)	21.71
3H	4.00±0.00 (2.24)	19.05	5.00±0.00 (2.45)	23.81	5.00±0.00 (2.45)	23.81	4.67±0.33 (2.35)	22.24
4H	3.67±0.33 (2.16)	17.48	4.67±0.33 (2.38)	22.24	4.67±0.33 (2.38)	22.24	4.33±0.33 (2.30)	20.62
5H	3.33±0.67 (2.07)	15.86	5.00±0.58 (2.44)	23.81	4.67±0.33 (2.38)	22.24	4.33±0.51 (2.30)	20.62
6H	2.67±0.33 (1.91)	12.71	2.33±0.33 (1.82)	11.09	2.33±0.33 (1.82)	11.09	2.44±0.11 (1.85)	11.62
7H	2.33±0.33 (1.82)	11.09	2.67±0.33 (1.91)	12.71	3.00±0.00 (2.00)	14.28	2.67±0.19 (1.91)	12.71
1NH	2.00±0.00 (1.73)	9.52	2.33±0.33 (1.82)	11.09	1.67±0.33 (1.63)	7.95	2.00±0.19 (1.73)	9.52
2NH	2.33±0.33 (1.82)	11.09	2.67±0.33 (1.91)	12.71	2.67±0.33 (1.91)	12.71	2.50±0.11 (1.88)	11.90
3NH	3.33±0.00 (2.00)	15.86	2.67±0.33 (1.91)	12.71	2.67±0.33 (1.91)	12.71	2.78±0.22 (1.94)	13.24
Mean	3.27 (2.05)	-	3.70 (2.15)	-	3.60 (2.12)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony	(0.15)
Time	(0.08)
Colony x Time	NS

Table 50. *Varroa destructor* inoculated brood cells only uncapped recorded at various time intervals after 20 h of inoculation during spring 2017

Col. No.	Cells only uncapped													
	20 h		22 h		24 h		26 h		28 h		30 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
2H	1.67±0.33 (1.63)	7.95	2.00±0.58 (1.72)	9.52	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.61±0.55 (1.22)	2.90
3H	1.67±0.33 (1.63)	7.95	1.67±0.33 (1.63)	7.95	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.56±0.50 (1.21)	2.67
4H	2.00±0.00 (1.73)	9.52	2.00±0.00 (1.73)	9.52	2.00±0.00 (1.73)	9.52	1.33±0.00 (1.52)	6.33	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	1.22±0.57 (1.45)	5.81
5H	1.00±0.00 (1.41)	4.76	1.33±0.33 (1.52)	6.33	1.33±0.33 (1.52)	6.33	1.33±0.33 (1.52)	0.00	1.00±0.00 (1.41)	4.76	1.00±0.00 (1.41)	4.76	1.11±0.29 (1.42)	5.28
6H	0.67±0.33 (1.28)	3.19	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.11±0.16 (1.05)	0.52
7H	0.67±0.33 (1.28)	3.19	0.67±0.33 (1.28)	3.19	0.33±0.33 (1.14)	1.57	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.39±0.19 (1.16)	1.86
1NH	1.00±0.00 (1.41)	4.76	1.33±0.33 (1.52)	6.33	2.00±0.00 (1.73)	9.52	1.00±0.00 (1.41)	4.76	1.33±0.33 (1.52)	6.33	1.33±0.33 (1.52)	6.33	1.33±0.21 (1.52)	6.33
2NH	2.00±0.00 (1.73)	9.52	2.00±0.00 (1.73)	9.52	2.00±0.00 (1.73)	9.52	2.00±0.00 (1.73)	9.52	1.67±0.33 (1.63)	7.95	2.00±0.00 (1.73)	9.52	1.94±0.08 (1.71)	9.24
3NH	2.00±0.00 (1.73)	9.52	3.00±0.00 (2.00)	14.28	3.00±0.00 (2.00)	14.28	3.00±0.00 (2.00)	14.28	3.00±0.00 (2.00)	14.28	3.00±0.00 (2.00)	14.28	2.83±0.24 (1.95)	13.48
Mean	1.27 (1.48)	-	1.3 (1.47)	-	1.0 (1.39)	-	0.73 (1.27)	-	0.73 (1.27)	--	0.77 (1.28)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformationLSD ($p=0.05$) for:

Colony (0.15)

Time (0.08)

Colony x Time NS

Table 51. Number of *Varroa destructor* inoculated brood cells only uncapped at various time intervals after 44 h of inoculation during spring 2017

Col. No.	Cells only uncapped							
	44 h		46 h		48 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
2H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
3H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
4H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
5H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
6H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
7H	0.33±0.33 (1.38)	1.57	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.11±0.08 (1.13)	0.52
1NH	0.00±0.00 (1.41)	0.00	0.00±0.00 (1.41)	0.00	0.00±0.00 (1.41)	0.00	0.00±0.00 (1.00)	0.00
2NH	0.00±0.00 (1.41)	0.00	0.00±0.00 (1.41)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
3NH	1.00±0.00 (1.41)	4.76	1.00±0.00 (1.41)	4.76	0.00±0.00 (1.00)	0.00	0.67±0.00 (1.28)	3.19
Mean	0.13 (1.05)	-	0.10 (1.04)	-	0.00 (1.00)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony	(0.15)
Time	(0.08)
Colony x Time	NS

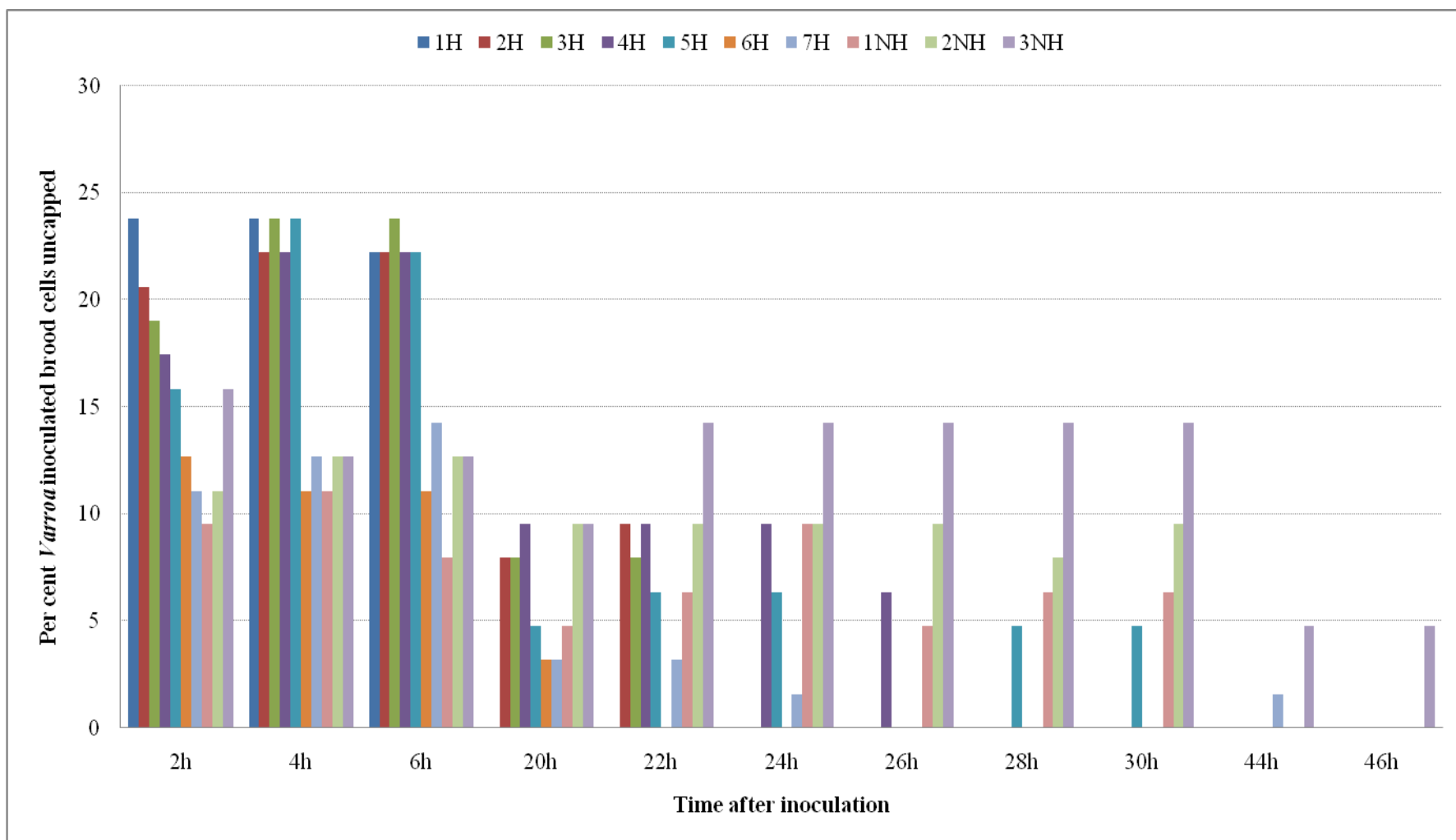


Fig. 36 Percentage of brood cells only uncapped in various test colonies at various time intervals after *Varroa* inoculation during spring 2017

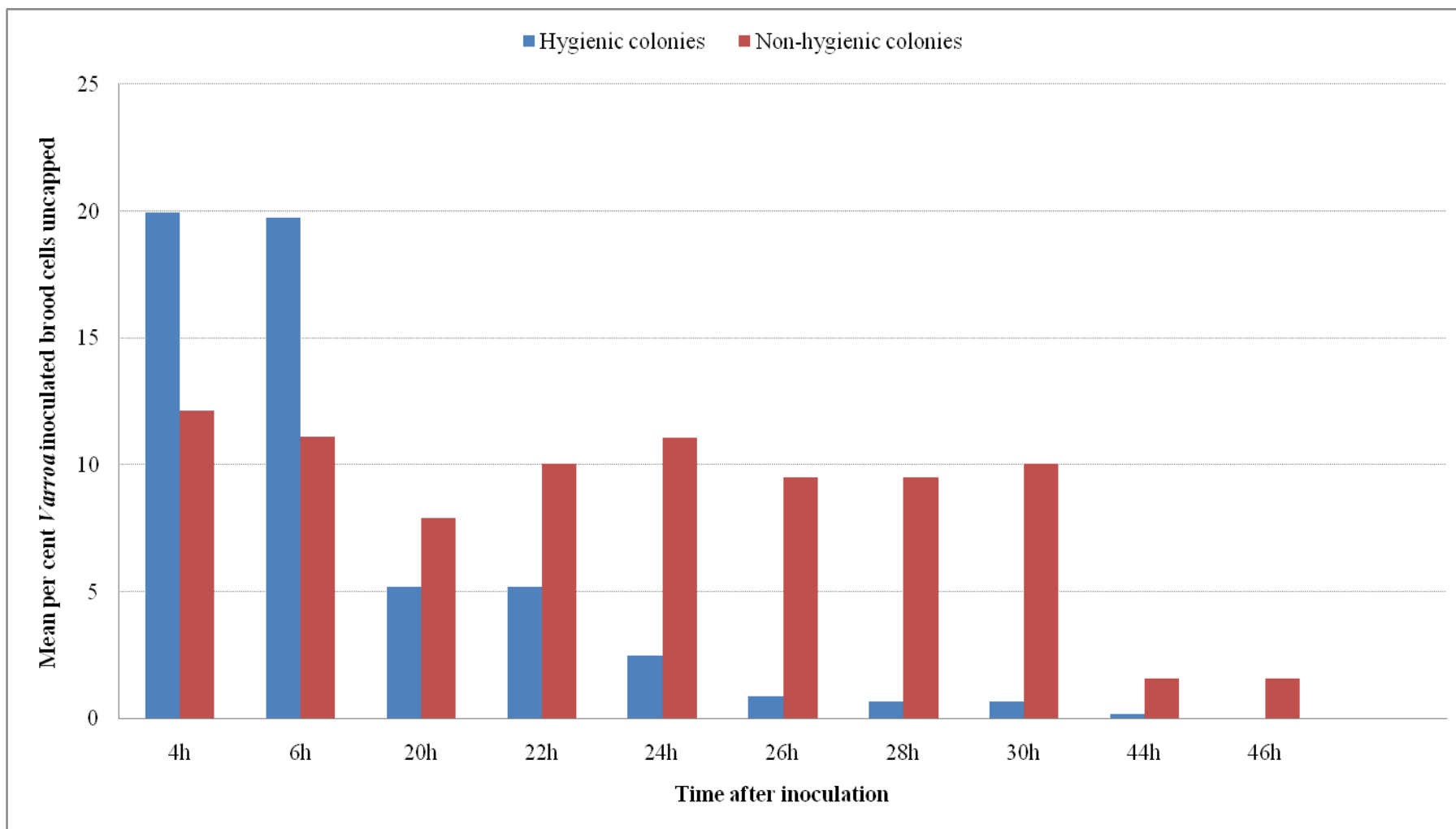


Fig. 37 Mean percentage of brood cells only uncapped in hygienic and non-hygienic colonies at various time intervals after *Varroa* inoculation during spring 2017

4.8.3.3 *V. destructor* inoculated brood cells remaining capped

The mean brood cells remaining capped after 2, 4 and 6 h of inoculation, were 79.13 (range: 73.00- 82.52%); 73.69 (range: 66.67-80.95%) and 73.01 per cent in the hygienic colonies and in non-hygienic colonies, the corresponding mean brood cells remaining capped were 88.36 (85.71-90.48%), 86.77 (85.71-88.90%) and 86.77 (85.71-88.90%), (Table 52, Fig. 38 and 39).

After 20 h, a mean of 5.66 (range: 1.57-14.28%) and 18.52 per cent (range: 14.28-22.24%) inoculated brood cells remained capped in the hygienic and non-hygienic colonies, respectively (Table 53, Fig. 38 and 39). After 24 h, no inoculated brood cells remained capped in hygienic colonies while, mean of 12.17 per cent capped brood was recorded in the non-hygienic colonies. After 28, 30 and 44 h, mean inoculated brood cells remaining capped in the non-hygienic colonies was 8.46, 6.87 and 1.59 per cent.

Though the hygienic colonies had achieved cent per cent opening of brood cells, the data was continued to be recorded for the non-hygienic colonies the next day after 48 h of inoculation till all the inoculated brood cells were uncapped and emptied and no brood cell remained capped (Table 54. Fig. 38 and 39).

4.8.4 Time interval to attain 100 per cent uncapping and cleaning of inoculated brood

After 24 h of inoculation, in colonies 1H, 2H, 3H and 6H, 100 per cent brood cells were emptied. In the next two hours (26 h) 100 per cent removal of inoculated brood cells in colony 7H and in the further next two hours (28 h) in colony 4H, cent per cent cleaning was achieved. In non-hygienic colonies, 1NH colony attained complete removal of inoculated brood after 46 h and colony 2NH and 3NH recorded 100 per cent cleaning of brood cells after 48 h. To achieve 100 per cent uncapping and cleaning of cells, the hygienic colonies on an average, took 25.71 h, whereas non-hygienic colonies took 47.36 h to achieve the same in the spring season.

It was observed that during the spring season, the process of uncapping and cleaning of *Varroa* inoculated brood cells was rapid than that recorded in autumn season. This may be due to the season, as in the spring season, there was very fast brood rearing activity and more number of brood cells were required for egg laying and storage and the population of the colonies too was strong as compared to that during the autumn season.

The present studies are in conformation with the studies conducted by various other authors. Removal of mite infested pupae was reported in *A. mellifera carnica* colonies (Boecking and Drescher 1991, 1992), and colonies of *A. mellifera ligustica* specifically bred for hygienic behaviour removed significantly more infested pupae than non-hygienic colonies (Spivak 1996). Thakur *et al* (1997) found that Carniolan bees in Germany removed 26-30 per cent of *Varroa*-infested worker brood. Guerra (2000) reported that Africanized colonies removed a significantly greater proportion of brood infested with *V. jacobsoni* Oudemans than did Italian colonies, headed by queens from the USA (59 vs. 31%, respectively).

Table 52. *Varroa destructor* inoculated brood cells remaining capped at various time intervals after the inoculations during spring 2017

Col. No.	Cells remaining capped							
	2 h		4 h		6 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	16.00±0.00 (4.12)	76.19	15.00±0.00 (4.00)	71.43	15.00±0.00 (4.00)	71.43	15.331±0.33 (4.04)	73.00
2H	16.33±0.33 (4.16)	77.76	15.33±0.33 (4.04)	73.00	15.00±0.00 (4.00)	71.43	15.56±0.40 (4.07)	74.10
3H	15.33±0.33 (4.04)	73.00	14.00±0.00 (3.87)	66.67	14.00±0.00 (3.87)	66.67	14.44±0.44 (3.93)	68.76
4H	17.00±0.58 (4.24)	80.95	15.33±0.33 (4.04)	73.00	15.33±0.33 (4.04)	73	15.89±0.56 (4.11)	75.67
5H	17.33±0.33 (4.28)	82.52	15.00±0.58 (4.00)	71.43	14.67±0.67 (3.96)	69.86	15.67±0.84 (4.08)	74.62
6H	17.33±0.33 (4.28)	82.52	17.00±0.00 (4.24)	80.95	17.00±0.00 (4.24)	80.95	17.11±0.11 (4.26)	81.48
7H	17.00±0.00 (4.24)	80.95	16.67±0.33 (4.20)	79.38	16.33±0.33 (4.16)	77.76	16.67±0.19 (4.20)	79.38
1NH	19.00±0.00 (4.47)	90.48	18.67±0.33 (4.43)	88.90	18.67±0.33 (4.43)	88.90	18.78±0.11 (4.38)	88.00
2NH	18.67±0.33 (4.43)	88.90	18.00±0.00 (4.36)	85.71	18.00±0.00 (4.36)	85.71	18.22±0.22 (4.38)	86.76
3NH	18.00±0.00 (4.36)	85.71	18.00±0.00 (4.36)	85.71	18.00±0.00 (4.36)	85.71	18.00±0.00 (4.36)	85.71
Mean	17. 20 (4.26)	-	16.30 (4.16)	-	16.20 (4.14)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony	(0.15)
Time	(0.08)
Colony x Time	NS

Table 53. *Varroa destructor* inoculated brood cells remaining capped recorded at various time intervals after 20 h of inoculations during spring 2017

Col. No.	Cells remaining capped													
	20 h		22 h		24 h		26 h		28 h		30 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	3.00±0.00 (2.00)	14.28	2.67±0.33 (1.91)	12.71	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.94±0.45 (1.32)	4.48
2H	1.00±0.00 (1.41)	4.76	0.67±0.33 (1.28)	3.19	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.28±0.26 (1.12)	1.33
3H	0.33±0.33 (1.14)	1.57	0.33±0.33 (1.14)	1.57	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.11±0.10 (1.05)	0.52
4H	1.67±0.33 (2.63)	7.95	1.33±0.33 (1.52)	6.33	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.50±0.44 (1.19)	2.38
5H	1.00±0.00 (1.41)	4.76	0.67±0.33 (1.28)	3.19	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.28±0.26 (1.12)	1.33
6H	1.00±0.00 (1.41)	4.76	0.67±0.33 (1.28)	3.19	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.28±0.26 (1.12)	1.33
7H	0.33±0.33 (1.14)	1.57	0.33±0.33 (1.14)	1.57	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.11±0.10 (1.05)	0.52
1NH	4.67±0.33 (2.38)	22.24	4.33±0.33 (2.31)	20.62	3.00±0.00 (2.00)	14.28	3.00±0.00 (2.00)	14.28	2.33±0.33 (1.82)	11.09	2.33±0.33 (1.82)	11.09	3.28±0.58 (2.05)	15.62
2NH	4.00±0.00 (2.24)	19.05	3.00±0.00 (2.00)	14.28	2.67±0.33 (1.91)	12.71	2.67±0.33 (1.91)	12.71	3.00±0.00 (2.00)	14.28	2.00±0.00 (1.73)	9.52	2.89±0.38 (1.96)	13.76
3NH	3.00±0.00 (2.00)	14.28	2.00±0.00 (1.73)	9.52	2.00±0.00 (1.73)	9.52	1.00±0.00 (1.41)	4.76	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	1.33±0.70 (1.48)	6.33
Mean	2.40 (1.78)	-	2.50 (1.67)	-	1.07 (1.36)	-	0.97 (1.33)	-	0.63 (1.22)	-	0.53 (1.20)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony	(0.15)
Time	(0.08)
Colony x Time	NS

Table 54. Number of *Varroa destructor* inoculated brood cells remaining capped recorded at various time intervals after 44 h of inoculation during spring 2017

Col. No.	Cells remaining capped							
	44 h		46 h		48 h		Mean	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
2H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
3H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
4H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
5H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
6H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
7H	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
1NH	0.67±0.33 (1.28)	3.19	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.22±0.16 (1.09)	1.05
2NH	0.33±0.33 (1.14)	1.57	0.33±0.33 (1.14)	1.57	0.00±0.00 (1.00)	0.00	0.22±0.10 (1.09)	1.05
3NH	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00	0.00±0.00 (1.00)	0.00
Mean	0.10 (1.04)	-	0.10 (1.04)	-	0.00 (1.00)	-	-	-

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

Figures in parentheses are means of $\sqrt{n+1}$ transformation

LSD (p=0.05) for:

Colony (0.15)

Time (0.08)

Colony x Time NS

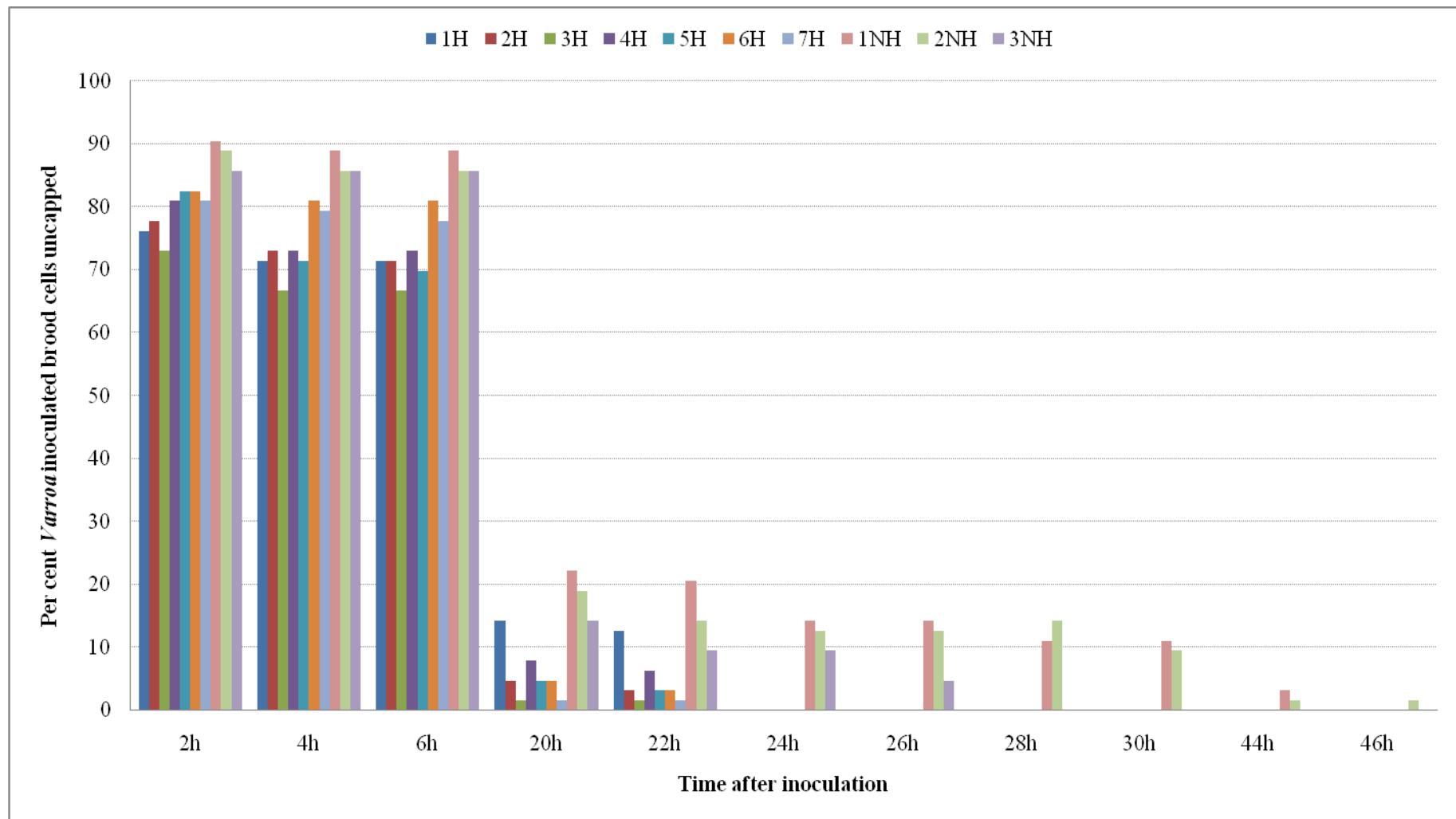


Fig. 38 Percentage of brood cells remaining capped in various test colonies at various time intervals after *Varroa* inoculation during spring 2017

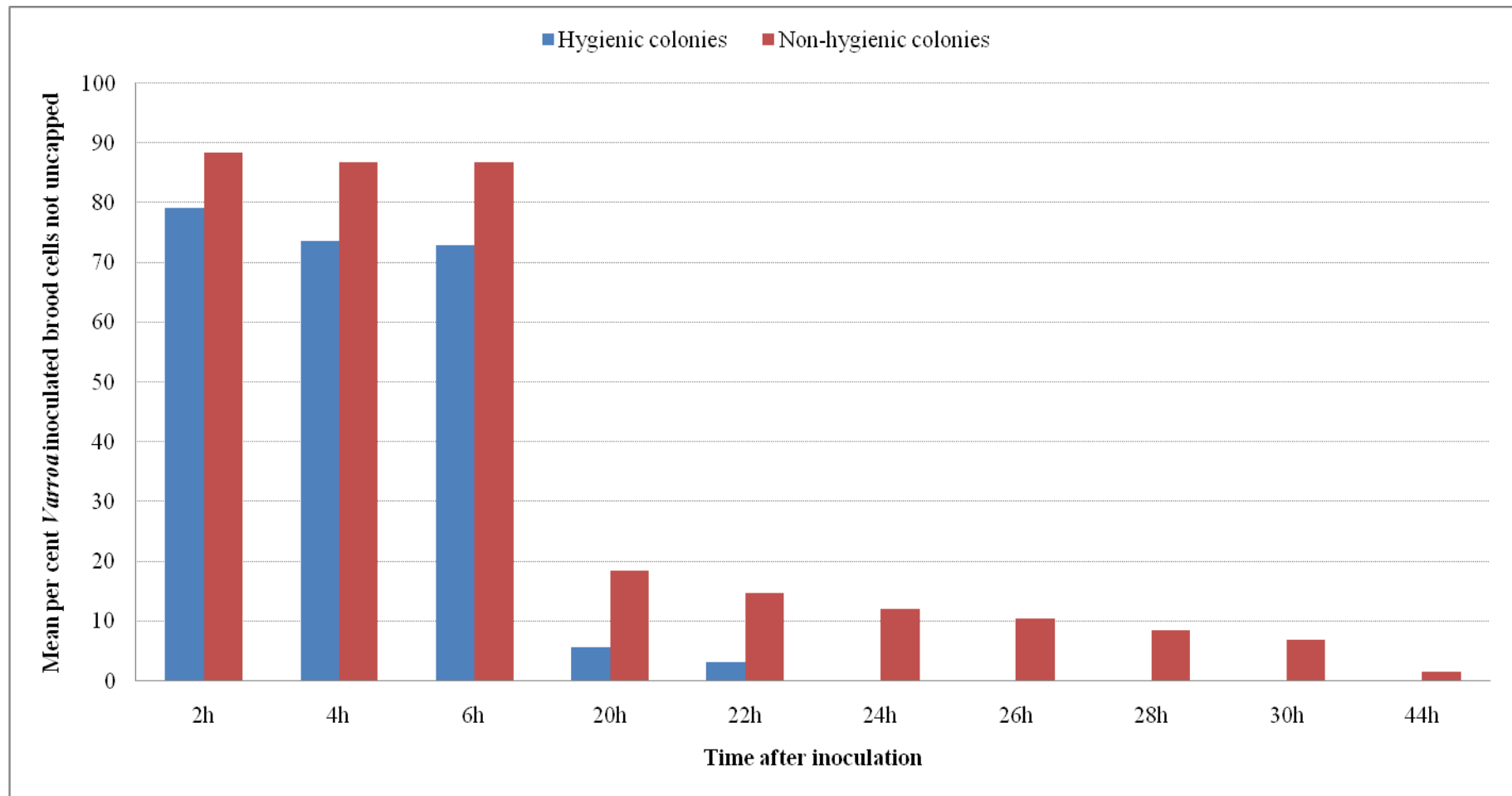


Fig. 39 Mean percentage of brood cells remaining capped in hygienic and non-hygienic colonies at various time intervals after *Varroa* inoculations during spring 2017

Hygienic bees removed the majority of mite infested brood at least 60 hours after the cell was sealed, which was after the mite had initiated oviposition (Donze *et al* 1996), when the bee is in the pupal stage (Spivak 1996). Removing the pupa at that time ensures destruction of any mite progeny. Zakar *et al* (2014) observed that removal of brood cells invaded by mites interrupted the reproductive cycle of *V. destructor*, prolonged its phoretic phase or killed the parasite. In cases of high infestation of *V. destructor*, the selection for hygienic behavior would decrease the reproduction rate of the mite (Wielewski *et al* 2012).

Uzunov *et al* (2014) suggested that since hygienic behaviour is mainly exhibited by workers that are younger than three weeks and different balance of young and old bees in the colony may affect expression of the trait at the colony level. This may, explain for the lower cleaning rate which was observed in the autumn as compared to that in the spring season.

As the colony characteristics are determined by the queen genotype (Bienefeld *et al* 2007), the availability of selected queens for production traits, as well as for hygienic behavior, contributes to increase the beekeeping productivity (Costa-Maia *et al* 2011) and to the lowest infestation rate of *V. destructor*.

CHAPTER V

SUMMARY

Apis mellifera Linnaeus is commonly known as the European, western or the common honey bee in different regions of the world and is largely hived for honey, pollen, beeswax, propolis, royal jelly and bee venom (Ratcliffe *et al* 2011). Besides being the major agent for global ecology service through pollination (Klein *et al* 2007, Allsopp *et al* 2008), it has been the main model of scientific social behavioural studies (Breed *et al* 2004, Solignac *et al* 2004, Beye *et al* 2006, The Honey Bee Genome Sequencing Consortium 2006). In addition, biologists are interested in the honey bees' social instincts and behavioural traits (Page *et al* 2000, The Honey Bee Genome Sequencing Consortium 2006, Shruthi *et al* 2009). They exhibit a wide variety of behavioral phenomena such as kin recognition, complex communication *via* the dance language, socially regulated division of labour, a large variety/ forms of learning, resistance to diseases and pests, hygienic behaviour, foraging and defensive behaviours that are of agricultural value which influence pollination and hive productivity either directly or indirectly (Hunt *et al* 1998, Oldroyd and Thompson 2007, Paleolog 2009, Shruthi *et al* 2009, Oxley *et al* 2010, Wray *et al* 2011). Most of the features of economic importance in beekeeping are the result of the behaviour of the whole colony.

Hygienic behaviour of honey bees provides multiple benefits for beekeepers with no apparent negative characteristics that accompany the trait. Selection and breeding for behaviour(s) of honey bees to control the most devastating brood diseases and mite pests are preferable to the application of chemicals (antibiotics and pesticides) into bee colonies which cause chemical contamination of bee products such as honey, pollen, propolis and wax.

Honey bees, in general, are exposed to a large diversity of microorganisms when compared with solitary insects, since the colonies of the former offer favourable ambient conditions for many parasites and pathogens, *inter-alia* also owing to a large amount of food and brood reserves like honey, pollen, wax and larvae (Schmid-Hempel 1998). Infestations and infections (bacterial, protozoan, fungal and viral) negatively affect colony growth and productivity, resulting in economic losses. The macro parasites of honey bees include mites viz., *Varroa destructor* Anderson and Trueman; *Acarapis woodi* Rennie and *Tropilaelaps clareae* Delfinado and Baker and pests like wax moths. Among these macro parasites, some additionally act as vectors of viruses and bacteria. The efficient hygienic behaviour increases resistance to various diseases, therefore, keeping bees having higher degree of hygienic behaviour is recommended as a natural method of minimizing the level of incidence of pests and diseases (Rinderer 1997, Palacio *et al* 2000). A well established bee breeding programme with the help of marker assisted selection for the production of high quality; pathogen and parasite resistant stocks will provide an alternative to chemical usage. Genetic markers that

could identify strong hygienic colonies in the field would prove to be a very useful tool for selecting colonies for breeding purpose and thus avoiding colonies that lack the hygienic marker.

Study of 'Evaluation and molecular characterization of hygienic traits in honey bee (*Apis mellifera* Linnaeus)' was carried out at the *Apis mellifera* Apiary, Insect Molecular Biology Laboratory of the Department of Entomology and at the School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana during February 2015 to April 2017. These studies included (i) screening of 100 individual colonies of *A. mellifera* for hygienic behaviour, (ii) molecular characterization of colonies using SSR markers reported to be linked with hygienic behaviour, (iii) candidate gene based markers to evaluate if there is variation in sequences of candidate genes between hygienic and non-hygienic colonies, (iv) temporal variation in hygienic behaviour response among hygienic and non-hygienic colonies, and (v) validation of screened hygienic colonies against *V. destructor* infestation. The present study is pioneering work in the country, wherein the information generated on hygienic behaviour in *A. mellifera* under Indian conditions would serve as baseline data for carrying out further studies on molecular characterization of hygienic traits.

Screening of *A. mellifera* colonies for hygienic traits

The colony level hygienic behaviour of 100 colonies was assessed by pin-killed brood method which involved measuring the rate at which a colony removes dead pupae that have been killed by pricking with an entomological pin. The experiment was run consecutively three times. Further this experiment was repeated for three alternate brood cycles i.e. at 6 weeks interval between each of the two consecutive test brood cycles, to determine the variability w.r.t. the sub-families in the colony.

Per cent killed brood removal

In the colonies screened, the dead brood removal after 24 h, ranged between 31.33-91 per cent in brood cycle I, whereas in brood cycle II and III it ranged between 44.00-92.67 and 55.67-95.00 per cent, respectively. Over the three test brood cycles, the mean pin-killed brood removal ranged between 46.78 to 91.00 per cent. After 48 h of pricking, the mean pin pricked brood cells emptied in brood cycle I, II and III ranged between 64.33-95.67, 64.00-95.67 and 70.00-97.67 per cent, respectively. The mean brood cells emptying across the three brood cycles ranged between 66.11 to 94.11 per cent. After 72 h, the mean pin killed brood cells emptying varied between 70.67- 95.67, 77.33-95.67 and 75.00-96.67 per cent in brood cycle I, II and III, respectively; the overall mean emptying of the brood ranged between 74.33-94.89 per cent. Perez-Sato *et al* (2009) assessed hygienic behaviour at colony level in 31 colonies and it ranged from 15 to 98 per cent. Sixty five per cent of colonies removed less

than 50% of freeze killed brood within 48 h and 22 per cent removed more than 70% and were thus classified as being hygienic.

Per cent uncapping of killed brood cells (without dead brood removal)

Mean percentage of uncapping of pricked brood cells (dead brood not removed) was also recorded. The data revealed that after 24 h of pricking, the mean percentage of uncapping of the pricked brood cells during the first test brood cycle ranged between 0.00-31.30, in alternate second test brood cycle it was 0.00-37.33 per cent, and in the third alternate test brood cycle, it ranged between 0.00-26.33 per cent. The overall mean uncapping of pin killed brood cells ranged between 1.44 to 22.22 per cent. After 48h, in brood cycle I, the maximum uncapping was 20.67 per cent, while in brood cycle II and III, it was 12.33 and 11.33 per cent, respectively. The overall mean for only uncapped brood was in the range of 0.44 to 10.44 per cent among the screened out colonies. In first test brood cycle, after 72 h, the maximum uncapping was 11.00 per cent while in the second and third alternate brood cycles, it was 11.67 and 15.33 per cent, respectively; over the three test brood cycles, the maximum uncapping was 11.67 per cent.

Per cent killed brood cells remaining capped

In the first brood cycle, the pin killed brood cells remaining capped after 24 h of the pricking ranged between 0.00-59.33 per cent. In the second and third alternate test brood cycles, the corresponding figures were 2.33-47.33 and 3.67-33.33 per cent. The overall mean of pin killed brood cells remaining capped ranged between 6.00-43.56 per cent. After 48 h, the cells of the first brood cycle remaining capped after killing ranged between 2.33-31.00 per cent and the corresponding figures for 2nd and 3rd alternate test brood cycles, these were in the range of 2.00-34.00 and 2.33- 28.00 per cent, respectively. The overall mean of brood cells remaining capped was reduced to minimum of 4.22 and a maximum of 31.00 per cent across the colonies that were screened. After 72 h, the brood remaining capped in the first, second and third test brood cycles ranged between 1.33-26.00, 0.67-22.67 and 1.00-20.33 per cent, respectively, with the mean over the brood cycles being 2.11-23.00 per cent

Grouping of colonies into different classes of hygienic behaviour

Out of the 100 colonies screened for hygienic behaviour, 45 were hygienic and 55 were non-hygienic; out of latter group, 42 were intermediary (71-80% emptying of pricked brood cells) and 13 were the least hygienic colonies (<71% emptying of pricked brood cells). The different classes of hygienic degree based on the expression of the hygienic traits displayed by adult honey bee workers in terms of the per cent brood removal were 41-50, 51-60, 61-70, 71-80, 81-90 and 91-100 per cent, respectively. After 24 h of pricking, one colony was in the group of 91-100 per cent hygienic, 44 in 81-90 per cent hygienic group, 42 in 71-80 per cent, 10 in 61-70 per cent range and three belonged to 41-50 per cent hygienic group.

After 48 h, the scenario of the number of colonies in different groups changed appreciably. Ninety five colonies (83 and 12) were in the two different groups i.e. 81-90 and 91- percent hygienic. Four colonies belonged to 71-80 per cent group and one colony was found in 61-70 per cent hygienic group. After 72 hours, majority of the colonies were found to have responded to mortality of brood; 64 colonies being in 81-90 and 34 colonies in 91-100 per cent hygienic group; only two colonies were in the intermediary class of 71-80 per cent.

Selection of colonies for molecular characterization of hygienic behaviour

From the 100 colonies screened for hygienic behaviour, seven colonies showing maximum percentage of hygienic trait after 24 h period of brood killing and three inferior most colonies, in this respect i.e. the most non-hygienic were selected for further evaluation and molecular characterization of hygienic behaviour. These seven most hygienic selected colonies were designated as 1H to 7H and the three non-hygienic colonies as 1NH to 3NH. After 24, 48 and 72 h, the hygienic behaviour ranged between 86.44-91.00, 92.11 to 94.11 and 92.89-94.89 per cent and 46.78- 47.33, 66.11-73.00 and 74.33-82.55 per cent in the 7 hygienic colonies and 3 non-hygienic colonies, respectively. The mean percentage of brood cells uncapped after 24, 48 and 72 h, ranged between 1.44 - 4.67, 0.22 -2.00 and 0.00-1.56 in the hygienic colonies and 9.56-20.89, 2.89 -10.44 and 3.44-4.78 per cent in the non-hygienic colonies. The brood remaining capped after 24, 48 and 72 h ranged between 6.00-12.78, 4.33-6.78 and 4.33-6.44 per cent in hygienic colonies and 32.33-43.56, 16.56 -31.00 and 14.00-23.00 per cent in non-hygienic colonies.

The selected 10 colonies (7 hygienic and 3 non-hygienic) were re-evaluated for hygienic behaviour to confirm the consistency w.r.t. to the expression of the hygienic traits. The methodology followed was the same as in the above experiment. The overall mean of dead brood removed after 24, 48 and 72 h in the hygienic colonies was 88.93, 93.28 and 94.21 per cent and 50.11, 72.89 and 81.33 per cent in the non-hygienic colonies. The mean of dead brood cells uncapped after 24, 48 and 72 h was 2.85, 1.28 and 0.52 per cent in hygienic colonies and 23.33, 6.74 and 1.89 per cent in the non-hygienic colonies. The pricked brood remaining capped after 24, 48 and 72 h, recorded a mean of 7.25, 6.42 and 5.27 per cent in the hygienic colonies and 26.63, 19.63 and 16.78 per cent in non-hygienic colonies. The present findings are in line with the studies conducted by Balhareth *et al* (2012) who reported that after 24, 48 and 72 h; the per cent general mean of dead brood removal in *A.m. jemenitica* was 85.28, 97.60 and 99.78 per cent.

Thus, from field studies, the seven hygienic and three non-hygienic selected colonies showed consistency in their hygienic traits and these colonies were used for collecting the samples of adult honey bee workers for genotyping with the SSR markers reported to be

linked with hygienic behaviour and the genetic diversity studies were also carried out on these selected colonies.

Molecular characterization of hygienic and non-hygienic *A. mellifera* colonies using SSR markers

Ten adult worker bee collected from each of the ten *A. mellifera* colonies headed by naturally mated queens (7 most hygienic and 3 most non-hygienic) at every six weeks interval between each of the two consecutive test brood cycles were genotyped at the 5 SSR loci reported to be linked with hygienic behaviour. K0263, A058 and K1601 were the nearest markers identified for the QTL *Hyg1*, *Hyg2* and *Hyg 3*, respectively. AT128 and K1601 were the nearest markers for the suggestive QTL responsible for uncapping component (*Uncap1* and *Uncap2*) and AC074 was the nearest marker for the removing component (*Rem1*). Based on the PCR amplification profiles of the genomic DNA of the worker honey bees of all the 10 colonies with the above markers, it was observed that these markers did not discriminate between the hygienic and non-hygienic colonies, and all the five markers showed polymorphism. The allelic richness (average number of alleles per locus) of markers K0263, A058, K1601, AT128 and AC074 ranged from 2-4 and all the markers were 100 per cent polymorphic. The diversity index, resolving power, marker index and PIC of all the five markers ranged between 0.37-0.80, 2.39-6.15, 0.83-2.41 and 0.23-0.60, respectively. The SSR markers linked with hygienic behaviour could not differentiate between the hygienic and non-hygienic trait as the colonies were headed by the naturally mated queens, and the colony consisted of population representing sisters and super sisters belonging to different patriline. Naturally mated queens yield quite different levels of intracolony genetic diversity, which affect overall colony performance.

Genetic diversity of *A. mellifera* colonies for hygienic traits using SSR markers

The 300 DNA samples, representing the 10 selected colonies, were PCR amplified with 40 SSR markers to study the genetic diversity both within the colonies and between the colonies. These 37 polymorphic markers produced a total of 137 scorable DNA fragments with an average of 3.7 fragments per marker of which 134 (97.81%) were polymorphic and three monomorphic (2.19%). The number of loci per marker ranged between two in markers UN368, A058, AC109 and K1610 and 7 in markers SV198 and 5983, respectively, while three markers produced six alleles each, one marker five alleles, 11 markers amplified four alleles each, 16 markers produced three alleles each. The size of the amplicons ranged between 80-450bp.

The PIC values, analyzed to characterize the capacity of each of the SSR markers to show polymorphic loci, ranged between 0.23 (K0263) to 0.77 (marker 5983). The average PIC value for all the evaluated loci was 0.52. Diversity index (DI)/ genetic diversity is the

expected heterozygosity. The maximum diversity index was 0.92 for the marker SV198 and the minimum of 0.37 was observed for the marker K0263. The mean DI value for all the evaluated markers was 0.68. Marker index (MI) is the capacity of each primer to detect polymorphism. The marker SV198 also recorded maximum marker index value and minimum was seen for the marker UN368 and the average MI value was 2.56. The Resolving power (Rp) ranged between 2.25 (B1293) and 6.30 (AP094) with a mean value of 3.4. Except for the three markers (UN368, K1610 and SV026), all other markers showed 100 per cent polymorphism.

The clustering pattern of the neighbour joining dendrogram representing 1H-7H hygienic colonies and 1NH- 3NH non-hygienic colonies of the first brood cycle formed three clusters. Cluster I comprised of four hygienic colonies, cluster II comprised two colonies each of hygienic and non-hygienic and cluster III consisted of one colony each of hygienic and non-hygienic. The dendrogram of the second alternate test brood cycle comprised of cluster I, the largest, consisting of five hygienic and one non-hygienic colony and also a small population belonging to colony 3H. The Cluster II consisted of 3NH and 3H colonies, both forming one group as in first test brood cycle. The cluster III consisted of 5H and 2NH colonies. The clustering pattern of the third test brood cycle also showed three clusters: cluster I comprising 3NH, 2H, 1H and 5H colonies, cluster II comprised 3H, 2NH, 1NH and 4H, and the cluster III comprised of 6H and 7H colonies; both these colonies consistently forming the same group though varying in cluster positions.

The neighbour joining dendrogram generated based on the scoring data of the amplification of the microsatellite loci in the 300 samples; the dendrogram comprised of three clusters: cluster I consisted of four hygienic colonies 7H, 6H, 1H and 2H; the colonies 5H, 2NH, 4H, 3H, and 1NH made up the cluster II, and the cluster III consisted of a single non-hygienic 3NH colony. The grouping pattern of the seven hygienic and three non-hygienic colonies varied in all the dendrograms constructed for the three test brood cycles and also in the dendrogram of all the 300 genotypes except for the colonies 6H and 7H which consistently formed one group, though present in varied clusters. The present findings are in corroboration of the studies conducted by Munoz *et al* (2008) where three types of pupae: pupae not parasitized, infested pupae with mites without reproductive success and infested pupae with mites with reproductive success were genotyped with five microsatellite loci and analysis revealed in the principal coordinate analysis, two main clusters of individuals, which consisted of 95.9% non-infested pupae and 68.8% infested pupae with mites without reproductive success in cluster-1 and 94.1% infested pupae with mites with reproductive success and 31.2% infested pupae with mites without reproductive success in cluster-2.

The similarity coefficient estimated by dice similarity coefficient in consecutive first, second and third test brood cycles ranged between 0.49-0.97, 0.46-0.97 and 0.50-0.97, respectively. Over the three test brood cycles, it ranged between 0.47-.98 with a mean value of 0.72. Hence, SSR markers used in the present study could generate genetic diversity between the colonies and thus, these SSR markers can be used in future for genetic diversity studies.

Molecular characterization of colonies using candidate gene specific primers

The 300 DNA samples, representing the 10 selected colonies, were used to amplify the candidate genes for hygienic behaviour with the gene specific primers. Seventeen candidate genes for hygienic behaviour were selected to study if these varied between the hygienic and non-hygienic colonies. Two primers per candidate gene were used to amplify genomic DNA of 10 colonies. It was observed that all the newly designed primers amplified all 300 individuals, generating amplicons of 450 – 550bp and no length polymorphism between the amplicons of hygienic and non- hygienic traits and intra colony variation was observed.

Sequencing of the candidate genes for hygienic traits

Among the 17 candidate genes studied in the previous experiments, five genes were selected, viz., *GB13839*, *GB12228*, *GB12301*, *GB 11135* and *GB 19509*. Four DNA samples each were taken from one most hygienic colony (6H) and from one non- hygienic colony (3NH). The DNA samples were PCR amplified with the primers of the five selected candidate genes. A small quantity of 5ul of the PCR product was resolved on 1.0 per cent agarose gel to confirm the amplification of the desired gene before sending for custom sequencing services.

The genes: *GB13839*, *GB 12228*, showed no sequence variation; thus these genes are totally conserved genes. The gene *GB 12301* showed sequence differences at several sites, but no variation was found between the hygienic and non-hygienic colonies. The gene *GB 19509* associated with olfactory behaviour, showed sequence differences at several sites indicating genetic differences at multiple sites; the variations were found at several sites and also between hygienic and non-hygienic trait. At position 282, Adenine occurs in the hygienic samples and Thymine in the non-hygienic samples; at position 309, Guanine is in the hygienic colony samples and Adenine in the non-hygienic samples; at 358, Guanine occurs in the hygienic samples and Thymine in the non-hygienic samples; at position 398, Cytosine is in the hygienic colony and Thymine in the non-hygienic colony; at 410, Cytosine in the hygienic colony and Adenine occurs in the non-hygienic colony; Adenine occurs in the hygienic samples and Cytosine occurs in the non-hygienic samples at position 460. Thus, in this gene, 6 differential SNPs were observed between hygienic and non-hygienic colonies. The present study suggests the presence of genetic variation which might be related to hygienic trait in

honey bees. Cornelissen (2015) reported the presence of genetic variation in *Dop3* and *Prohormone-1*, which might be related to resistance in honey bees against *Varroa*.

Hygienic response among the hygienic and non-hygienic colonies

The colony level hygienic behaviour of the selected 10 colonies was assessed at every 2h interval following pricking of brood. It was observed that after 2 h, no cleaning of brood cells was done. After 6 h, the mean of brood cells cleaned was 17.35 and 8.00 per cent in hygienic and non-hygienic colonies, respectively; after 20 h, it was 86.97 per cent and 39.6 per cent; after 22 h, 90.13 and 44.15 per cent; after 24 h, 91.40 and 47.63 per cent; at 26, 28 and 30 h these were 92.75, 93.32 and 93.35 and 53.30, 68.96 and 73.52 per cent in the hygienic colonies and non-hygienic colonies, respectively; 44 h after pricking, the mean dead brood clearing was 93.70 and 80.6 per cent; after 46 and 48 h, it was 94.36 and 94.47; and 83.54 and 86.93 per cent, in the hygienic and non-hygienic colonies, respectively. At 50 h onwards, no change was observed in the hygienic colonies, while in the non-hygienic colonies, a mean of 88.55 per cent brood clearing was recorded. The various studies conducted also reveal that hygienic bees remove a greater number of dead brood compared to non-hygienic within the same timeframe (Spivak and Gilliam 1993, Kefuss *et al* 1996, Spivak and Downey 1998, Arathi and Spivak 2001, Panasiuk *et al* 2008, Bāk *et al* 2010).

Time interval to attain hygienic colony grade (> 80% hygienic behaviour)

The hygienic colonies took an average of 20.6 h to exhibit the hygienic grade (>80% pricked brood removal) while the non-hygienic colonies took 44.6 h to achieve the same.

After 2 hours, the mean dead brood cells only uncapped (without brood removal) was 14.11 and 8.40 per cent in the hygienic colonies and non-hygienic colonies; after 4 and 6 h, 5.25 and 17.34 per cent ; 2.74 and 8.00 per cent, respectively. After 20 h, the mean uncapping was reduced to 7.23 per cent in the hygienic colonies, as by this time, these colonies had reached >80 per cent cleaning of the cells. In non-hygienic colonies; as the adult worker honey bees were slow in detecting the cues from the pricked brood cells, the mean brood cells only uncapped was 40.13 per cent. After 24 h, the mean was 4.62 per cent in the hygienic colonies and 39.22 per cent in non-hygienic colonies; after 26, 28 and 30 h of pricking, the mean brood cells only uncapped were 4.21; 3.91 and 3.86 per cent and 36.48, 23.19 and 18.67 per cent in hygienic and non hygienic colonies, respectively; at 44 h, the corresponding values were 4.83 and 12.67 per cent; at 52 h, 3.22 and 5.85 per cent.

In the initial hours of treatment i.e. at 2 , 4 and 6 h, the mean brood cells remaining capped were 85.69, 80.27 and 73.87 per cent and 91.59, 86.85 and 81.74 per cent in hygienic and non-hygienic colonies, respectively; after 20 h, the corresponding values were 6.06 and 20.22 per cent in hygienic and non-hygienic colonies, respectively. By this time period, the hygienic colonies had expressed hygienic traits, i.e. >80.00 per cent cells cleaning, thus

resulting in low per cent of capped brood cells remaining. In the hygienic colonies; after 26 h, the mean brood cells remaining capped were 2.98 per cent after 48h, 1.89 per cent, with no further change till the data was recorded. In the non-hygienic colonies, the mean brood cells remaining capped after 22, 24, 26, 28 and 30 h were 17.29, 13.11, 9.67, 7.96 and 7.22 per cent, respectively, and after 44, 46, 48 and 50 h it was 6.74, 6.44, 5.78 and 5.63 per cent, respectively. After this time period till 72 h of treatment no change was observed.

Further research can focus on gene expression studies of various genes for identifying which of the genes are responsible for variation in hygienic behaviour traits. As one of the gene showed differential SNPs between hygienic and non-hygienic colonies, SNP based markers can be designed and studied to find if any of it is associated with hygienic behaviour.

Validation of markers

The five SSR markers reported to be linked with the hygienic behaviour when amplified in the queens of the selected 10 colonies (7 hygienic and 3 non-hygienic) showed no variation for the markers between the hygienic and the non-hygienic colonies.

Validation of screened hygienic colonies against *V. destructor* infestation

Varroa mites were used in inoculating worker larval brood near capping. Single mite was inoculated in each brood cell. The experiment validation was conducted during autumn and spring breeding seasons. In total, 21 inoculations (three groups of seven brood cells each) were made in every test colony, thrice in succession. The total time period between inoculation and brood removal was recorded.

Autumn 2016

During autumn experiment, the mean of *Varroa* mite inoculated brood cells emptied for the seven hygienic colonies, after 2, 4 and 6 h was 1.36, 3.17 and 5.66 per cent, respectively. For the three non-hygienic colonies the mean was 0.52 and 2.11 per cent after 4 and 6 h, respectively. After 2h no work was started in non-hygienic colonies. After 20 h, the mean removal of the mite inoculated brood emptied was 82.76 and 49.73 per cent in hygienic and non-colonies, respectively; and after 24 h, the corresponding values were 93.43 and 61.90 per cent. After 30 h of inoculation, six hygienic colonies had achieved 100 per cent brood removal while in non-hygienic colonies; it was in the range of 71.83-85.71 per cent. After 44 h, all the hygienic colonies had achieved 100 per cent brood removal and in non-hygienic colonies, a mean of 94.70 per cent was observed. The non-hygienic colonies recorded 100 per cent removal of inoculated brood after 52 h of inoculation.

The mean per cent of *Varroa* inoculated brood cells only uncapped after 2, 4 and 6 h was 14.52, 17.90 and 18.59 per cent and 4.76, 6.35 and 6.85 per cent in hygienic and non-hygienic colonies, respectively. After 20 h, the mean brood cells uncapped was 3.62 and 1.59

per cent in the hygienic and non-hygienic colonies. After 24 h, the corresponding values were 4.3 and 2.11 per cent.

The mean brood cells remaining capped after 2, 4 and 6 h was 84.12, 78.68 and 75.97 per cent and 95.24, 93.13 and 92.08 per cent in the hygienic and non-hygienic colonies, respectively. After 20 h, a mean of 13.14 and 48.68 per cent inoculated brood cells remained capped in the hygienic and non-hygienic colonies, respectively; after 24 h, the average was 2.72 and 34.39 per cent; after 28 h, all inoculated cells had opened, while an average of 19.57 per cent mite inoculated brood cells were still capped in the non-hygienic colonies. To achieve 100 per cent uncapping and cleaning of cells, the hygienic colonies on an average took 28h whereas non-hygienic colonies took 50.67 h to achieve the same in autumn season.

Spring 2017

During spring experiment, the mean *Varroa* mite inoculated brood cells emptied for the seven hygienic colonies, was 3.62, 6.57 and 7.25 per cent after 2, 4 and 6 h, respectively, and it was 1.57 and 2.11 per cent after 4 and 6 h, respectively in the non-hygienic colonies. After 24 h, a mean of 95.24 and 77.24 per cent of the infested brood was removed in hygienic and non-hygienic colonies, respectively; after 30 h of inoculation, six of the hygienic colonies had achieved 100 per cent infested brood removal and in non-hygienic colonies, it was in the range of 80.95-85.71 per cent; after 44 h, all the hygienic colonies had achieved 100 per cent removal of inoculated brood while in non-hygienic group of colonies, it was 95.24 per cent. The non-hygienic colonies recorded 100 per cent removal of inoculated brood after 52 h of inoculation.

For the hygienic colonies, the mean uncapping of inoculated cells (non brood removal) was 17.23, 19.96 and 19.73 per cent, while in the non-hygienic colonies; it was 12.16, 12.17 and 11.12 per cent after 2, 4 and 6 h, respectively. After 20 h, the mean uncapping was 5.22 and 7.93 per cent in the hygienic and non- hygienic colonies, respectively; after 44 h, the corresponding figures were 0.22 and 10.04 per cent.

The mean brood cells remaining capped after 2, 4 and 6 h were 79.13, 73.69 and 73.01 per cent in the hygienic colonies, respectively, and in non-hygienic colonies, the corresponding capping was 88.36, 86.77 and 86.77 per cent. After 20 h, a mean of 5.66 and 18.52 per of inoculated brood cells remained capped in the hygienic and non-hygienic colonies, respectively; after 24 h, mean of 12.17 per cent of brood cells were still capped in the non-hygienic colonies. The present studies are supported by the studies conducted by Spivak (1996) who reported that *A. m. ligustica* Spinola colonies particularly bred for hygienic behavior, removed significantly more infested pupae than non hygienic colonies and Thakur *et al* (1997) found that Carniolan bees in Germany removed 26-30% of *Varroa*-infested worker brood.

To achieve 100 per cent uncapping and cleaning of cells, the hygienic colonies on an average took 25.71 h whereas non-hygienic colonies took 47.36 h to achieve the same. It was observed that during spring season, the process of uncapping and cleaning of Varroa inoculated brood cells was rapid than that in the autumn season. This may be due to very fast brood rearing activity in the spring season, and also more number of brood cells were required for egg laying and storage and the population of the colonies too was strong as compared to that during the autumn season.

CONCLUSIONS

1. Out of the 100 colonies screened for evaluation of their hygienic behaviour traits, 45 colonies were hygienic and 55 were non-hygienic out of which 42 were intermediary (71-80% removal of pricked brood) and 13 were the least hygienic colonies (<71% emptying of pricked brood cells).
2. Within 24 h of brood pricking, mean brood cells emptying was 83.65 per cent (range: 80.22-91.0 %) in 45 colonies while it was 73.05 per cent (range: 46.78-79.56%) in the remaining 55 colonies.
3. Within 24 h, in the seven selected most hygienic colonies, a mean of 91.40 per cent of brood cells removal was recorded (range: 87.22-94.89%), while in the 3 most non-hygienic colonies, the mean brood removal was only 47.63 per cent (range: 43.56-52.44%).
4. The 5 SSR markers reported to be linked with hygienic behaviour did not differentiate the colonies into hygienic and non-hygienic groups.
5. Out of the 40 SSR markers used to study the genetic diversity between the hygienic and non-hygienic colonies, 37 markers were polymorphic. The diversity index value of the 37 SSR markers ranged between 0.37-0.92, marker index value between 0.24-6.41 and the PIC value ranged between 0.23-0.77.
6. The clustering pattern between the hygienic and non-hygienic colonies in each alternate brood cycle produced by neighbour-joining dendrogram showed that the 10 colonies (7 most hygienic and 3 most non-hygienic) divided into 3 clusters with further divisions into sub clusters and groups and sub-groups. The clustering pattern between the seven hygienic and the three non-hygienic colonies in all the three brood cycles showed three clusters. Cluster I consisted of four hygienic colonies, cluster II consisted of 3 hygienic and 2 non-hygienic and cluster III consisted of one non hygienic colony. The grouping pattern of the hygienic and non-hygienic colonies varied in all the three brood cycles except in the colonies 6H and 7H which consistently formed one group.

7. The newly designed 34 gene specific primers amplified all 300 genomic DNA samples from both types of colonies and no length polymorphism was observed between the hygienic and non-hygienic individual DNA samples.
8. Sequencing of the PCR amplicons in three candidate genes revealed no variation, thus showing that these are totally conserved genes. One gene *GB19509* showed six differential SNPs between hygienic and non-hygienic colonies. The SNP based markers can be designed and validated on large populations to find if these SNPs are associated with hygienic behaviour.
9. The 7 hygienic colonies took an average of 20.6 h to exhibit the hygienic trait (>80% pricked brood removal) while the non-hygienic colonies took 44.6 h to achieve this level.
10. Within 24 h of *V. destructor* inoculation of the brood cells in the seven selected hygienic colonies, a mean of 93.43 per cent of brood removal was recorded (range: 85.71-100%), while in the 3 non-hygienic colonies, the mean brood removal was only 61.90 per cent (range: 53.95-69.86%) in the autumn season. To achieve cent per cent uncapping and cleaning of cells, the hygienic colonies on an average, took 28 h whereas non-hygienic colonies took 50.67 h.
11. Within 24 h of *V. destructor* inoculation of the brood cells in the seven selected hygienic colonies during the spring season, a mean of 95.23 per cent of brood cells removal was recorded (range: 88.90-100%), while in the 3 non-hygienic colonies, the mean brood removal was only 77.24 per cent (range: 76.19-77.76%). During this season, the hygienic colonies group took an average of 25.71 h and non-hygienic colonies took 47.33 h to obtain cent per cent of removal of the infested brood.

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LIST OF PUBLISHED/ACCEPTED/SUBMITTED RESEARCH ARTICLES

S. No.	Title	Journal	NAAS rating	Status
1	Differential hygienic trait characteristics between hygienic and non-hygienic <i>Apis mellifera</i> Linnaeus colonies	Current Science	6.97	Submitted
2	Molecular variability of <i>Apis mellifera</i> Linnaeus colonies selected for hygienic behaviour using microsatellite markers	Journal of Apicultural Research	8.08	Submitted
3	Validation of hygienic colonies (<i>Apis mellifera</i> Linnaeus) against the infestation by <i>Varroa destructor</i> Anderson and Trueman	Indian Journal of Experimental Biology	7.17	Submitted

From: **Chandrika Ramesh** <currsci@ias.ac.in>
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Differential hygienic trait characteristics between hygienic and non-hygienic *Apis mellifera* Linnaeus colonies

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ABSTRACT

The hygienic behaviour of 100 colonies was assessed by pin killed brood method. Out of the 100 colonies screened for hygienic behaviour, 45 colonies were hygienic, 55 were non-hygienic out of which 42 were intermediary (71-80% emptying of pricked brood cells) and 13 were least hygienic colonies (<71% emptying of pricked brood cells). The mean percentage of brood cells emptied after 24 h of pricking in hygienic, intermediate hygienic and non-hygienic colonies was 83.65, 75.71 and 58.00 per cent. the data recorded after every 2 h in 10 colonies revealed that after 2h no cleaning of brood cells was recorded, whereas after 6 h, 17.35 and 8.00 per cent in hygienic and non- hygienic colonies, respectively. After 24 h it was 91.40 and 47.63 per cent in the hygienic and non-hygienic colonies, respectively. The hygienic colonies took on an average 20.6 h to exhibit the hygienic behaviour (>80% pricked brood removal) while the non-hygienic colonies took 44.6 h as this trait is associated with resistance to pests and diseases it should be considered in selection programs in queen breeders' apiaries.

Keywords: honey bee, hygienic behavior, naturally mated queen, pin killed brood method, temporal variation

INTRODUCTION

The honey bees are eusocial insects and are important not only for the honey they produce; but are also the most dominant pollinators of agricultural and horticultural crops. It is estimated that 80 per cent of flowering plants, 90 per cent of all horticultural and vegetable crops, especially hybrids and three quarters of staple crop plants that feed human kind, depend upon insect pollination to attain potential yield¹. Bees also play an important role in conserving the biodiversity and thereby integrity of global ecosystems². Saraswat (2011) reported that there are nearly 1.8 million hive honey bee colonies in India and the total honey production including that from wild honey bees is estimated at 65,000 tonnes³. During the year 2015-2016, the Punjab is the leading state in the country in honey production, accounting for 40 per cent of the nation's apiary honey production⁴.

The honey bee, *Apis mellifera* Linnaeus, commonly known as European, western or the common honey bee is largely domesticated for honey, pollen, beeswax, propolis, royal jelly and bee venom⁵. Besides being the major agent for pollination⁶, it has been the main model of scientific social behavioral studies^{7, 8}. It exhibits a wide variety of behavioral phenomena such as kin recognition, complex communication via the dance language, socially regulated division of labour, and large variety forms of learning, resistance to diseases and pests, hygienic behaviour, foraging and defensive behaviors which are of agricultural value as they influence pollination and hive productivity either directly or indirectly^{9,10,11}. Most of the features of economic importance in beekeeping are the result of the behaviour of the whole colony which is composed of a queen, a few hundred drones and worker

bees. A queen usually copulates with 10 to 17 drones, thus the colony is a super-family with a common queen mother and sub-families that have different drones as fathers¹².

The social behaviour traits of the colony traits are quantitative in nature and are a result of genetic and environment interactions⁹. Since the colonies of the honey bees offer favourable ambient conditions owing to a large amount of food and brood reserves like honey, pollen, wax and larvae¹³, they are exposed to a large diversity of microorganisms. The regulation of temperature and humidity as well as the presence of many closely related and regularly interacting individuals in the colony provide ideal conditions for growth of parasites and microbial pathogens. Infestations and infections negatively affect colony growth and productivity, resulting in economic losses.

Hygienic behaviour has evolved as a general mechanism of resistance to brood diseases, and provides significant resistance to a number of commercially important pathogens including *Ascosphaera apis* (the causative agent of chalkbrood), *Paenibacillus larvae larvae* (American Foulbrood) and *Varroa mites*^{14, 15, 16}. It is a two-step process carried out by some worker honey bees upon detection of dead or diseased larvae or pupae in brood comb. The process first involves 'uncapping' (elimination of the wax cap covering the brood cell), followed by 'removal' of the corpse inside¹⁷. The efficient hygienic behaviour increases resistance to various diseases, limits the multiplication and spread of infestation. Therefore, keeping bees having higher degree of hygienic behaviour is recommended as a natural method of minimizing the level of incidence of pests and diseases¹⁸ and the colonies that express this behaviour are economically important to beekeepers¹⁵. The present studies were proposed to evaluate the hygienic traits among the honey bee colonies and the temporal variation in hygienic behaviour response among hygienic and non-hygienic colonies.

Materials and methods

Screening of honey bee colonies for hygienic behaviour

The studies were conducted at the *A. mellifera* Apiary, Department of Entomology, Punjab Agricultural University, Ludhiana. Colony-level hygienic behaviour of 100 colonies was assessed by pin killed brood method wherein 100 sealed worker brood cells at pink eye pupal phase in the test comb, were pierced using number 1 entomological pin¹⁹. After perforating the brood, the combs were marked for identifying them and were returned into the colonies. The degree of hygienic behaviour (HB) was determined as a percentage of cleaned cells after piercing the brood²⁰. The percentage of brood removal in each colony was recorded after 24, 48 and 72 h and three replications were performed. The experiment was further repeated for 3 alternate brood cycles i.e. at 6 weeks interval between each of the two consecutive test brood cycles. The multiple tests were performed to account for the variability in sub-families w.r.t. their hygienic behaviour.

Temporal variation in hygienic behaviour response

Ten colonies (7 hygienic and 3 non-hygienic) were selected from the 100 colonies screened for hygienic behaviour. The same methodology was followed in the 10 selected colonies and the observations were recorded at every 2 h interval for three days.

Statistical analysis

The data were analyzed using Analysis of Variance (ANOVA) for finding the significance of difference among the colonies w.r.t. their hygienic trait and were separated by least significant difference (LSD) at $p=0.05$ level²¹.

Results and discussion

Expression of hygienic behaviour after 24, 48 and 72 h in colonies screened

In the 100 colonies screened, after 24, 48 and 72 h of pricking; the data recorded on the mean percentage of dead brood cells emptied, ranged between 46.78-91.00; 66.11-94.11 and 74.33-94.89 per cent, whereas, the mean percentage of pricked brood cells only uncapped (dead brood not removed) ranged between 1.44 -22.22; 0.44-10.44, and 0.00-11.67 per cent and the mean percentage of pin killed brood cells remaining capped ranged between 6.00-43.56; 4.22-31.00 and 2.11-23.00 per cent, respectively. Out of the 100 colonies screened for hygienic behaviour, 45 colonies were hygienic, 55 were non-hygienic out of which 42 were intermediary (71-80% emptying of pricked brood cells) and 13 were least hygienic colonies (<71% emptying of pricked brood cells). After 48 and 72 h number of hygienic, intermediate and non-hygienic colonies was 95, 4 and 1 and 97, 2 and 1, respectively (Fig. 1). The mean percentage of brood cells emptied after 24h of pricking in hygienic, intermediate hygienic and non-hygienic colonies was 83.65, 75.71 and 58.00 per cent and after 48 and 72h the hygienic expression increased in the respective groups of colonies as given in Fig. 2.

The present findings are in line with the findings of the studies conducted by different scientists^{22, 23, 24, 25}. After 24 48 and 72h, the per cent hygienic behaviour was 85.28, 97.6 and 99.78; 43.84, 79.32 and 93.29 per cent in *A.m. jemenitica* and *A. m. carnica*, respectively²⁶. The mean of dead brood uncapped and removed within 24 and 48 h by the worker honey bees in infected colonies is significantly greater than in the healthy colonies and in infected colonies in addition to resistance; it reduces the spread of diseases and parasitic infections²⁷. Previous studies have suggested that the detection and subsequent removal of diseased larvae are guided by olfactory stimuli²⁸. A response threshold model was postulated to describe the differences in colony- level expression of the behavior²⁵. Bigio *et al*²⁹ also observed that colonies headed by naturally-mated queens had high levels of hygiene (57.8 – 100 %). The present findings also corroborate with the findings of Nouredine and Haddad³⁰, who conducted hygienic behaviour studies in 40 colonies of Algerian local bees *A. mellifera intermissa* and observed that it is characterized by a good cleaning ability (91 .56 per cent in spring and, 83.5 per cent in autumn). The mean percentage of dead brood removed and uncapped after 24 and 48 hours was 37.57 and 90.39 per cent in healthy colonies and 41.21 and 98.28 per cent in infected colonies³¹.

Expression of hygienic behaviour at every 2 h interval

It was observed that 2 h after pricking of brood cells mean percentage of dead brood removal in all the colonies was negligible as it ranged between 0 to 1.11 per cent. After 6 h, the mean percentage of brood removal was 17.35 and 8.00 per cent in hygienic and non- hygienic colonies,

respectively. After 20 and 22 h mean percentage of the pricked brood cells emptied in the hygienic colonies was 86.97 and 90.13 per cent and 39.6 and 44.15 per cent in the non-hygienic colonies. After 24 h the mean brood cells emptied was 91.40 and 47.63 per cent in the hygienic and non-hygienic colonies, respectively. At 26, 28 and 30 h of treatment the mean pricked brood cells emptied were in the range of 92.75; 93.32 and 93.35 and 53.30, 68.96 and 73.52 per cent in the hygienic colonies and non-hygienic colonies, respectively. After 44 h, the mean brood cells emptied was 93.7 and 80.6 per cent in hygienic and non-hygienic colonies, respectively. In the hygienic colonies the mean dead brood cells emptied after 46 and 48 h was 94.36 and 94.47 per cent, respectively. At 50 h onwards no change was observed in the hygienic behaviour of the hygienic colonies. While in the non-hygienic colonies during the same time period, the mean dead brood cells emptied were 83.54 and 86.93, respectively, and it kept on increasing till 54 h after treatment, where a mean of 88.55 per cent was recorded. The data was recorded till 72 h after treatment but no increase was observed in the dead brood cells being emptied.

Time period required to attain hygienic behaviour (>80 per cent)

From the data recorded it is observed that the hygienic colonies on an average took 20.6 h to exhibit the hygienic behaviour (>80% pricked brood removal) while the non-hygienic colonies took 44.6h to reach >80%. Thus from this study it is inferred that, without disturbing the hives normal activities the hygienic trait of colony can be assessed 24 h after treatment. The present findings are in line with that of Pereira *et al.*³², who reported that one hour after perforation of the brood cells, hygienic bees had removed 43%, while the non-hygienic bees removed only 25%. Hygienic bees completed the removal in 15 h, whereas the non-hygienic bees took over 30 h. A total of 403 colonies from three regions were tested and observed that in the first 4 h, on average, colonies had detected at least 50% of the perforated cells. Four hours after perforation, all the colonies had already some fully uncapped cells. At 6 h, colonies displayed a wide range of detected cells (32 to 100%), uncapped cells (0 to 100%) and cleaned cells (0 to 82%)³³. The frozen brood was removed within 46.9 h and 76.5 h after introduction from the hygienic and non-hygienic colonies³⁴.

Conclusions

As this trait is heritable, breeding queens for hygienic behaviour is beneficial for commercial beekeeping and it is also possible. Thus this trait should be considered in selection programs in queen breeders' apiaries. Out of the 100 colonies screened for hygienic behaviour, 45 colonies were hygienic, 55 were non-hygienic out of which 42 were intermediary and 13 were least hygienic colonies. The mean percentage of brood cells emptied after 24 h of pricking in hygienic, intermediate hygienic and non-hygienic colonies was 83.65, 75.71 and 58.00 per cent. The data recorded after every 2 h in 10 colonies revealed that in initial hours no cleaning of the brood cells was observed. The hygienic colonies on an average took 20.6 h to exhibit the hygienic behaviour while the non-hygienic colonies took 44.6 h. Thus the hygienic colonies identified can be used as breeders' colonies for queen rearing to increase the frequency of the hygienic trait in further populations.

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Table 1. Mean percentage of brood cells emptied after 2 h of pricking in hygienic and non-hygienic colonies

Col No.	2h	4h	6h	20h	22h	24h	26h	28h	30h	44h	46h	48h
1H	1.11±0.80 (3.38)	8.89±1.57 (17.21)	16.89±1.06 (24.22)	90.22±0.11 (71.75)	92.11±0.22 (73.67)	94.33±0.19 (76.21)	96.11±0.22 (78.64)	96.33±0.00 (78.97)	96.33±0.00 (78.97)	97.00±0.00 (79.99)	97.00±0.00 (79.99)	97.00±0.00 (79.99)
2H	0.33±0.33 (1.54)	6.78±2.89 (13.18)	19.44±1.39 (26.12)	92.67±0.49 (74.36)	93.33±0.51 (75.06)	94.56±0.22 (76.50)	95.78±0.80 (78.26)	96.00±0.67 (78.56)	96.00±0.67 (78.56)	96.00±0.67 (78.54)	96.00±0.67 (78.54)	96.00±0.67 (78.54)
3H	0.00±0.00 (0.00)	8.78±0.11 (17.11)	19.44±0.40 (26.14)	93.22±0.62 (74.92)	94.89±0.49 (76.96)	94.89±0.49 (76.96)	95.11±0.49 (77.26)	95.89±0.59 (78.38)	95.78±0.49 (78.23)	95.89±0.59 (78.35)	95.89±0.59 (78.35)	95.89±0.59 (78.35)
4H	0.22±0.22 (0.90)	3.11±0.87 (9.45)	15.44±0.29 (23.12)	81.89±1.43 (64.83)	84.11±2.16 (66.59)	87.89±3.02 (69.95)	89.78±2.23 (71.57)	89.78±2.23 (71.57)	89.78±2.23 (71.57)	90.56±1.53 (72.25)	92.22±0.80 (73.85)	92.33±0.83 (73.98)
5H	0.00±0.00 (0.00)	4.00±0.69 (10.83)	15.67±0.84 (23.29)	80.89±2.57 (64.17)	85.56±2.00 (67.76)	87.22±2.31 (69.21)	88.78±2.23 (70.60)	90.22±1.06 (71.83)	90.56±0.91 (72.13)	90.56±0.91 (72.13)	91.33±0.69 (72.89)	91.78±0.28 (73.33)
6H	0.00±0.00 (0.00)	3.56±0.40 (10.74)	19.11±0.78 (25.89)	83.44±0.91 (66.03)	88.22±2.19 (70.09)	90.67±0.38 (72.22)	92.67±1.26 (74.38)	93.00±1.26 (74.77)	93.00±1.26 (74.77)	93.33±0.95 (75.13)	93.78±0.91 (75.63)	94.00±0.68 (75.87)
7H	0.00±0.00 (0.00)	5.89±0.62 (13.88)	15.44±1.72 (23.05)	86.44±1.28 (68.42)	91.66.33±1.86 (72.94)	93.56±4.08 (74.87)	91.00±3.37 (73.18)	92.00±2.53 (74.03)	92.00±2.53 (74.03)	92.44±2.51 (74.57)	94.33±1.36 (76.47)	94.33±1.36 (76.47)
1NH	0.00±0.00 (0.00)	3.44±1.49 (8.68)	6.89±1.35 (14.95)	33.22±3.02 (35.13)	41.11±1.64 (39.86)	43.56±1.49 (41.28)	50.78±4.26 (45.43)	67.56±3.13 (55.30)	70.78±5.39 (57.45)	75.22±6.77 (60.51)	81.11±2.55 (64.31)	85.89±3.25 (68.18)
2NH	0.00±0.00 (0.00)	2.22±1.90 (5.62)	9.44±2.12 (17.65)	40.78±2.11 (39.66)	44.11±2.23 (41.59)	46.89±2.61 (43.19)	53.11±1.79 (46.77)	66.89±2.96 (54.89)	75.00±1.90 (60.01)	83.56±6.19 (66.74)	85.67±4.10 (68.13)	87.33±3.91 (69.46)
3NH	0.00±0.00 (0.00)	2.56±1.93 (6.66)	7.67±1.76 (15.84)	44.89±2.81 (42.04)	47.22±3.61 (43.38)	52.44±2.26 (46.39)	56.00±2.00 (48.43)	72.11±1.44 (58.12)	74.78±2.86 (59.91)	83.11±4.65 (66.09)	83.89±3.90 (66.61)	87.56±1.44 (69.39)
LSD (p=0.05)	(1.82)	(2.89)	(1.19)	(1.24)	(1.11)	(1.11)	(1.03)	(1.13)	(1.22)	(1.32)	(1.08)	(1.12)

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

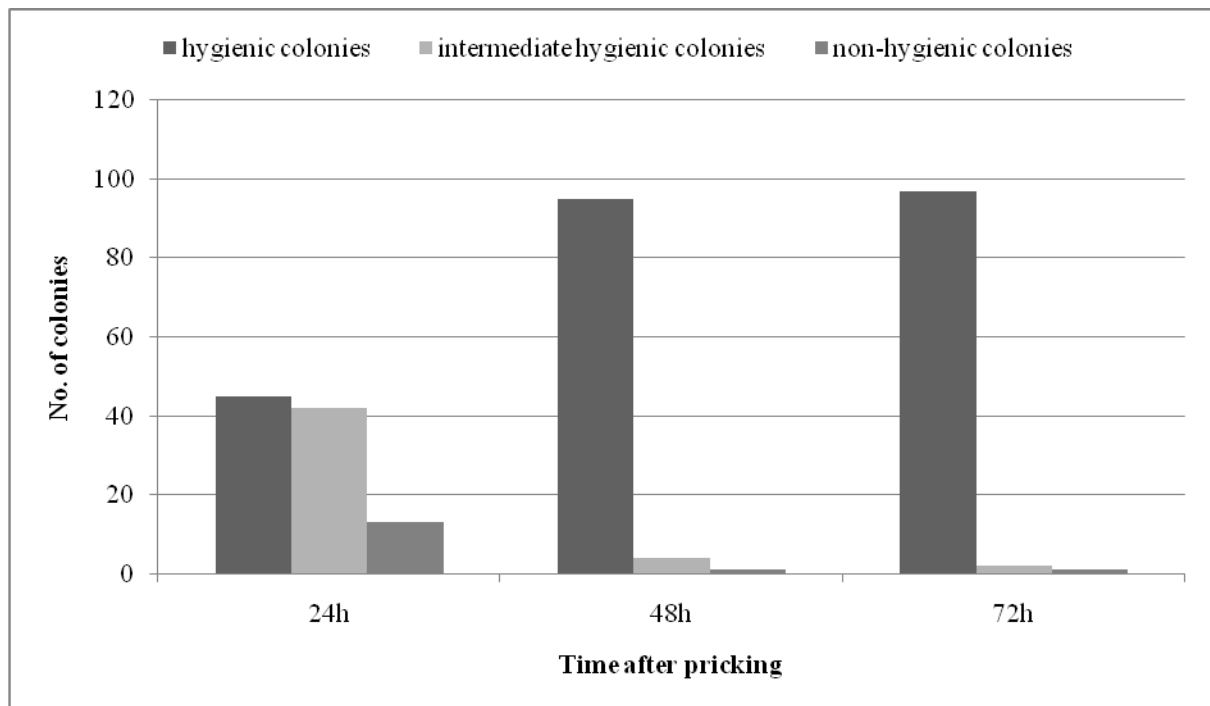


Figure 1. Number of colonies expressing hygienic behaviour after 24, 48 and 72 h of pricking

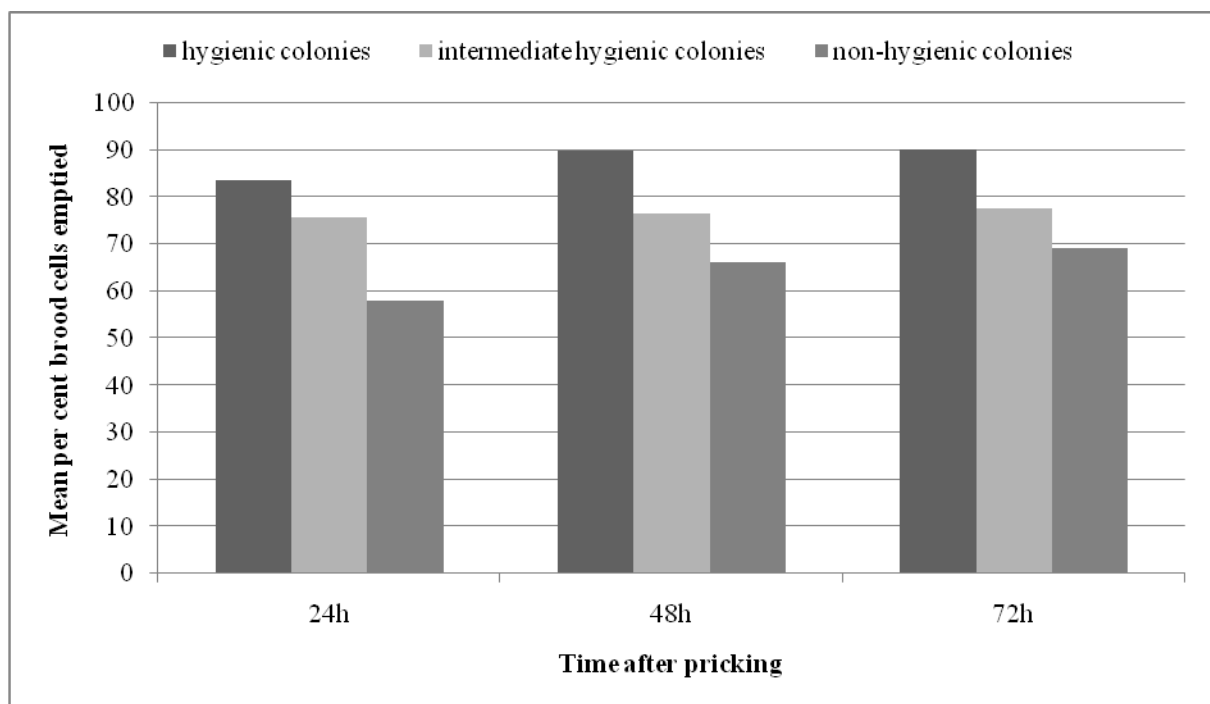


Figure 2. Mean per cent of brood cells emptied after 24, 48 and 72 h of pricking in hygienic, intermediate hygienic and non-hygienic colonies

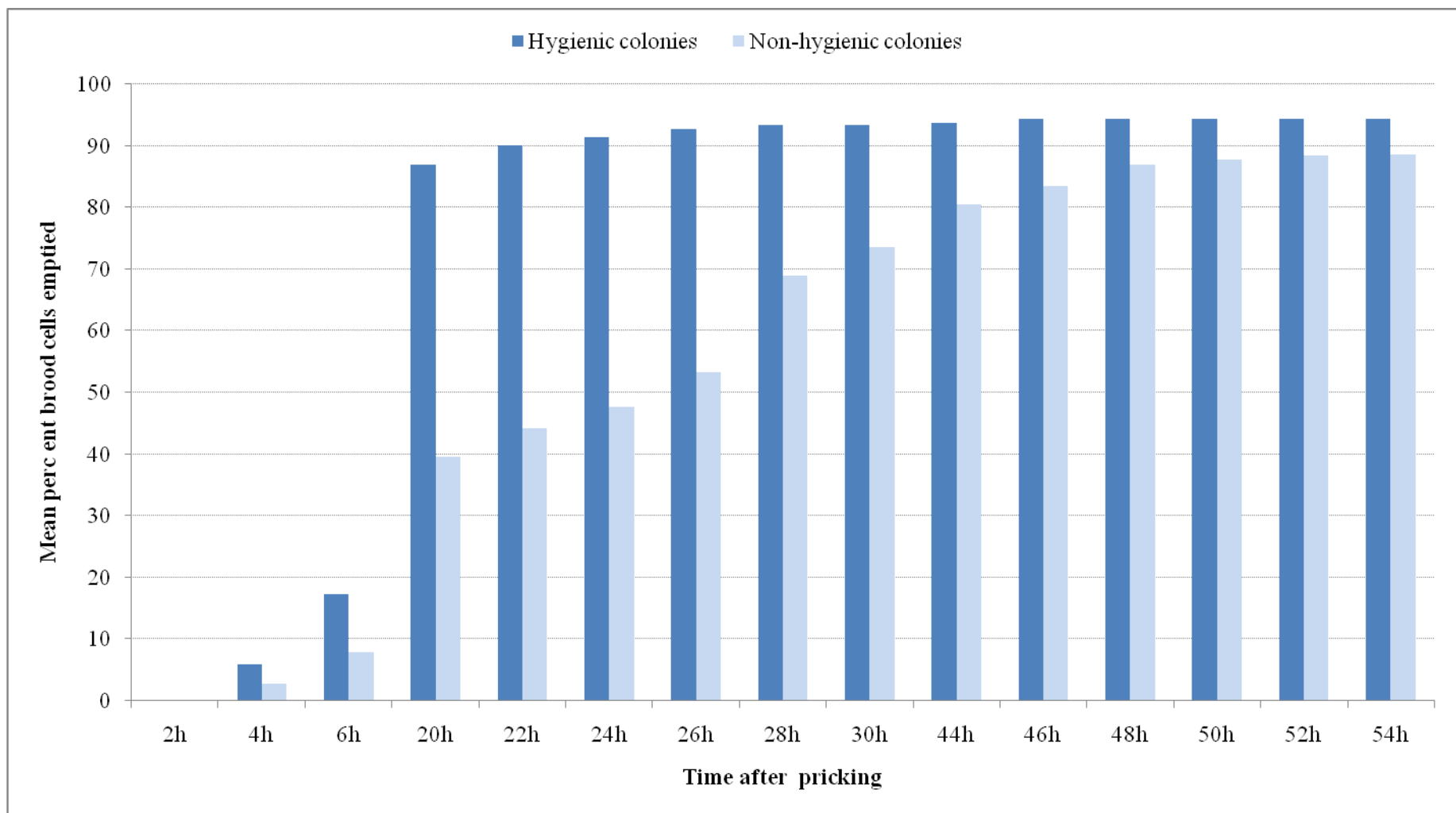


Figure 3. Mean percentage of brood cells emptied after 2 h of pricking in hygienic and non-hygienic colonies

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Validation of hygienic colonies (*Apis mellifera* Linnaeus) against the infestation by *Varroa destructor* Anderson and Trueman

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ABSTRACT

The ten colonies (7 hygienic and 3 non-hygienic) were validated against *Varroa destructor* infestation for the two seasons; autumn and spring. During autumn, in the seven hygienic colonies, the mean of *Varroa* mite inoculated brood cells emptied after 2, 4 and 6 h was 1.36, 3.17 and 5.66 per cent, while, in the non-hygienic colonies it was 0, 0.52 and 2.11 per cent, respectively. After 24 h, a mean of, 93.43 per cent of brood cells removal was recorded, in the hygienic colonies, while in the non-hygienic colonies, it was only 61.90 per cent. During spring season, in the hygienic colonies, the mean of inoculated brood cells emptied after 2, 4 and 6 h were 3.62, 6.57 and 7.25 per cent, respectively while in the non-hygienic colonies the mean was 0 per cent, 1.57 and 2.11 per cent. After 20 h, it ranged between 82.52-95.24 per cent and 71.43-76.19 per cent in the hygienic and non-hygienic colonies, respectively. In the autumn season, the hygienic colonies on an average took 28 h, whereas non-hygienic colonies took 50.67 h to achieve 100 per cent uncapping and cleaning of cells. On the other hand, the hygienic colonies on an average took 25.71 h, whereas non-hygienic colonies took 47.36 h to achieve the same in the spring season. In cases of high infestation of *V. destructor*, the selection for hygienic behavior would decrease the reproduction rate of the mite.

Keywords: defense, honey bee, hygienic behavior, temporal variation, *Varroa destructor*

The *Varroa* mite's natural host is the Asian hive bee, *Apis cerana*. Fabricius. Damage to Asian honey bee colonies is rarely experienced since a stable host-parasite relationship has been developed over a long evolutionary period¹. Such a relationship is distinguishably lacking in the European honey bee. The infestation of *A. mellifera* by *V. destructor* was reported to originate nearly half a century ago², when the *A. mellifera* colonies were brought into contact with *A. cerana*. It is an obligate ectoparasite and feeds on the haemolymph of adult bees and the brood. For reproduction, it chooses only the capped worker and drone brood of *A. mellifera*, and only the drone brood of *A. cerana*. On the other hand, the mortality in *A. mellifera* colonies due to *V. destructor* can reach up to 100 per cent within two to five years, if timely and proper mites control measures are not taken³. Besides, high mite populations were also observed to be associated with increased incidences of viral infections, lower weight at hatching, and shortened life span of the adult bees⁴, as well as deformed wing and shortened abdomen.

Hygienic behaviour has the potential to limit the population growth of *Varroa* in three ways: i) The immature mites are killed when the pupa is removed, which decreases the average number of offspring per reproducing mite, ii) the phoretic period of adult female mites is extended that survive the removal of the pupae, and iii) the mortality of the adult mites increases if they are damaged by the adult bees through grooming when they escape through the opened cell⁵. Therefore, keeping bees having higher degree of hygienic behaviour is recommended as a natural method of minimizing the incidence of pests and diseases as infestations and infections

negatively affect colony growth and productivity, resulting in economic losses⁶. The present study was conducted to assess the hygienic behaviour as defense against *V. destructor* so that colonies expressing resistance to the mite population can be selected in breeding experiments.

Materials and methods

The studies were conducted at the *Apis mellifera* Apiary at Entomological Research Farm, Department of Entomology, Punjab Agricultural University, Ludhiana. The ten colonies (7 hygienic and 3 non-hygienic) were validated against *V. destructor* infestation for the two seasons; autumn, 2016 and spring, 2017. *V. destructor* adult mites were collected from infested worker brood and drone brood using Varroa fork and from adult bees using 'sugar roll method'. The mites were used in inoculating worker larval brood near capping. In total, 21 inoculations (three groups of seven brood cells each) were made in every test colony, thrice in succession. The observations were recorded at every 2 h interval till the complete removal of mite infested brood. The time period between inoculation and uncapping and brood removal was also recorded. The data were analyzed using Analysis of Variance (ANOVA) for finding the significance of difference among the colonies w.r.t. their hygienic trait and were separated by least significant difference (LSD) at $p=0.05$ level⁷. The data on hygienic behaviour were transformed using $\sqrt{n+1}$ transformation.

Results and discussion

***V. destructor* inoculated brood cells emptied after various time intervals during autumn season, 2016**

In the seven hygienic colonies, the mean of *Varroa* mite inoculated brood cells emptied after 2, 4 and 6 h was 1.36, 3.17 and 5.66 per cent, respectively, while, in the non-hygienic colonies it was 0, 0.52 and 2.11 per cent. After 20 h of inoculation, in four of the hygienic colonies the percentage of inoculated brood cells emptied ranged between 85.71-88.90 per cent and in the non-hygienic colonies it was in the range of 42.86-58.71 per cent. After 24 h, in the selected hygienic colonies, a mean of 93.43 per cent of brood cells removal was recorded, while in the non-hygienic colonies, it was only 61.90 per cent. After 30 h of inoculation, six hygienic colonies have achieved 100 per cent emptiness of inoculated brood cells and in non-hygienic colonies it was in the range of 71.83-85.71 per cent. After 44 h all the hygienic colonies have reached 100 per cent removal of inoculated brood cells and in non-hygienic colonies a mean of 94.7 per cent was observed (Table 1 and Fig. 1).

***V. destructor* inoculated brood cells emptied after various time intervals during spring season, 2017**

During spring season, in the seven hygienic colonies, the mean of *Varroa* mite inoculated brood cells emptied after 2, 4 and 6 h was 3.62, 6.57 and 7.25 per cent, respectively while in the non-hygienic colonies the mean was 0 per cent, 1.57 and 2.11 per cent. In the hygienic colonies, the percentage of inoculated brood cells emptied ranged between 82.52- 95.24 per cent after 20 h of

inoculation and in the non- hygienic colonies at this hour, the brood cells emptied were in the range of 71.43-76.19 per cent. After 24 h of inoculated brood cells, in the selected hygienic colonies, a mean of 95.24 per cent of brood cells removal was recorded, while in the non-hygienic colonies, it was 77.24 per cent. After 30 h of inoculation, six hygienic colonies have achieved 100 per cent emptiness of inoculated brood cells and in non-hygienic colonies it was in the range of 80.95-85.71 per cent. After 44 h all the hygienic colonies have reached 100 per cent removal of inoculated brood cells and in non-hygienic colonies it was a mean of 95.24 per cent. The non-hygienic colonies recorded 100 per cent removal of inoculated brood cells after 52 h of inoculation (Table 2 and Fig. 2).

Time interval to attain 100 per cent uncapping and cleaning of inoculated brood cells

In the autumn season the hygienic colonies on an average took 28 h whereas non-hygienic colonies took 50.67 h to achieve 100 per cent uncapping and cleaning of cells. On the other hand, the hygienic colonies on an average took 25.71 h whereas non-hygienic colonies took 47.36 h to achieve the same in spring season.

It was observed that during spring season, the process of uncapping and cleaning of *Varroa* inoculated brood cells was rapid than that recorded in autumn season. This may be due to the season, wherein in spring season there is brood build up activity and more number of brood cells are required for egg laying and storage and the population of the colonies is strong as compared to autumn season.

The present studies, are in conformation with the studies conducted by various authors. In European or western honey bees, some colonies of *A. mellifera carnica* Pollmann detected, uncapped, and removed mite-infested pupae⁸. Colonies of *A. m. ligustica* Spinola specifically bred for hygienic behavior removed significantly more infested pupae than non hygienic colonies⁵. Carniolan bees in Germany removed 26-30 per cent of *Varroa*-infested worker brood⁹. Africanized colonies removed a significantly greater proportion of brood infested with the parasitic mite *Varroa jacobsoni* Oudemans¹⁰. There was a significant negative correlation between hygienic behavior and the numbers of *Varroa* in the colonies¹¹⁻¹². Hygienic bees removed the majority of mite infested brood at least 60 h after the cell is sealed, which is after the mite has initiated oviposition³. Removing the pupa at that time ensures destruction of any mite progeny. Removal of brood cells invaded by mites interrupts the reproductive cycle of *Varroa destructor*, prolongs its phoretic phase or kills the parasite¹³. During good nectar flow bees remove dead brood faster, thereby preparing cells for nectar collection¹⁴. Since hygienic behaviour is mainly exhibited by workers that are younger than three weeks¹⁵⁻¹⁷ and different balance of young and old bees in the colony may affect expression of the trait at the colony level. This may, explain for the lower cleaning rate which was observed in autumn as compared to in spring season¹⁸.

Conclusions

Thus, from the studies conducted it was concluded that the hygienic colonies were effective in removing the pupae infested with *Varroa* mite and hygienic behaviour can be used as a defense against the *Varroa* mite. It was also observed that during the spring season more percentage of *Varroa* infested brood cells were cleaned as compared to the autumn season and 100 per cent cleaning of the cells was achieved in 25.71 h in hygienic colonies whereas the bees took 47.36 h to achieve the same in non-hygienic colonies. The selection for hygienic behaviour against infestation of *V. destructor* would reduce the mite population, encouraging implications for beekeeping because it shows that a breeding programme for hygiene is highly promising as breeding hygienic bees will avoid/reduce the use of chemicals in beekeeping. The high levels of hygiene shown for the naturally-mated queens present a positive picture for this trait.

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Table 1. Mean percentage of *Varroa destructor* inoculated brood cells emptied at various time intervals during autumn 2016

Colony No.	6h	20h	22h	24h	26h	28h	30h	44h	46h	48h	50h	52h
1H	9.52	77.76	80.95	88.90	100	100	100	100	100	100	100	100
2H	4.76	85.71	92.05	100	100	100	100	100	100	100	100	100
3H	6.33	87.29	87.29	93.67	100	100	100	100	100	100	100	100
4H	4.76	76.19	77.76	85.71	92.05	100	100	100	100	100	100	100
5H	4.76	76.19	80.95	85.71	90.48	95.24	95.24	100	100	100	100	100
6H	4.76	88.90	96.81	100	100	100	100	100	100	100	100	100
7H	4.76	87.29	88.90	100	100	100	100	100	100	100	100	100
1NH	3.19	42.86	42.86	52.38	53.95	68.24	71.83	92.05	92.05	95.24	95.24	100
2NH	1.57	47.62	57.14	61.90	74.62	84.14	84.14	96.81	96.81	100	100	100
3NH	1.57	58.71	58.71	69.86	73	85.71	85.71	95.24	95.24	100	100	100

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

H: Hygienic

NH: Non-hygienic

H: Hours

Table 2. Mean percentage of *Varroa destructor* inoculated brood cells emptied at various time intervals during spring 2017

Colony No.	2h	4h	6h	20h	22h	24h	26h	28h	30h	44h	46h	48h
1H	0.00	4.76	6.33	85.71	87.28	100.00	100.00	100.00	100.00	100.00	100.00	100.00
2H	1.57	4.76	6.33	87.28	87.28	100.00	100.00	100.00	100.00	100.00	100.00	100.00
3H	7.95	9.52	9.52	90.48	90.48	100.00	100.00	100.00	100.00	100.00	100.00	100.00
4H	1.57	6.33	6.33	82.52	84.14	88.90	93.67	100.00	100.00	100.00	100.00	100.00
5H	1.57	4.76	6.33	90.48	90.48	93.67	93.67	95.24	95.24	100.00	100.00	100.00
6H	4.76	7.95	7.95	92.05	96.81	100.00	100.00	100.00	100.00	100.00	100.00	100.00
7H	7.95	7.95	7.95	95.24	95.24	98.43	100.00	100.00	100.00	98.43	100.00	100.00
1NH	0.00	1.57	3.19	73.00	73.00	76.19	80.95	82.52	82.52	92.05	100.00	100.00
2NH	0.00	1.57	1.57	71.43	76.19	77.76	77.76	77.76	80.95	98.43	98.43	100.00
3NH	0.00	1.57	1.57	76.19	77.76	77.76	80.95	85.71	85.71	95.24	95.24	100.00

Data values represent mean of 3 replications

21 inoculations were made in every test colony, thrice in succession

H: Hygienic

NH: Non-hygienic

H: Hours

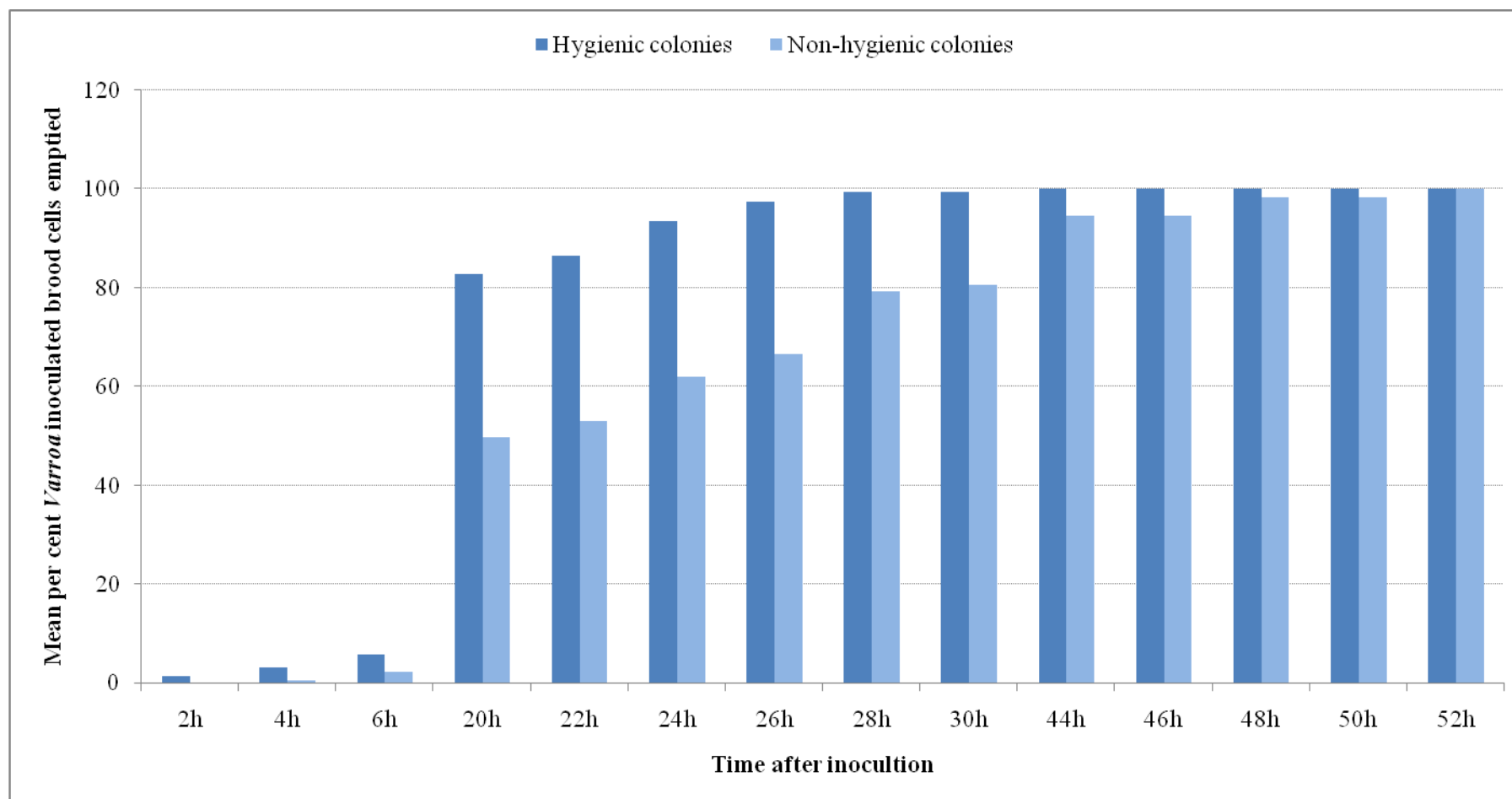


Figure 1. Mean percentage of brood cells emptied in hygienic and non-hygienic colonies after various time intervals of *Varroa* inoculation during autumn 2016

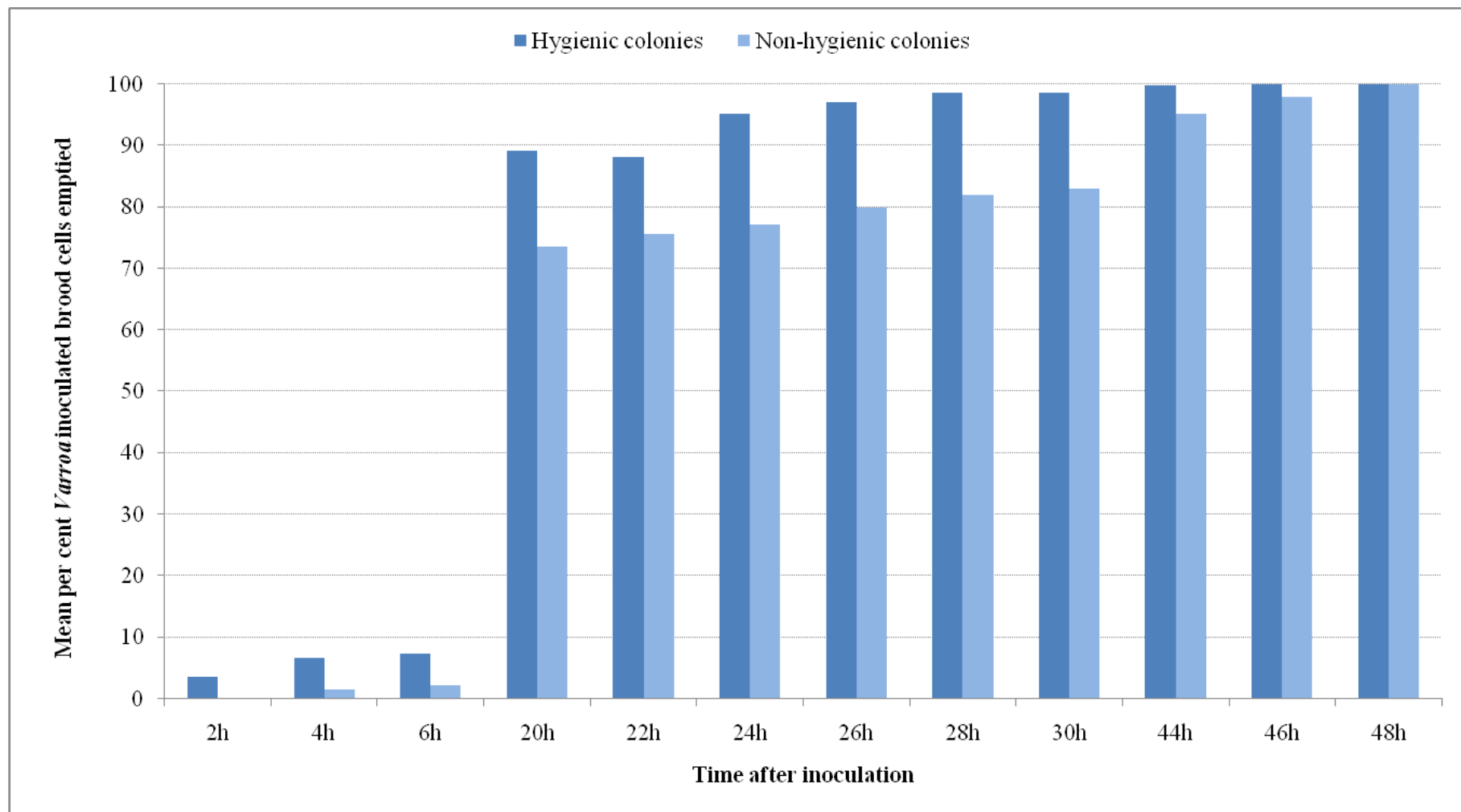


Figure 2. Mean percentage of brood cells emptied in hygienic and non-hygienic colonies after various time intervals of *Varroa* inoculation during spring 2017

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**MOLECULAR VARIABILITY OF *APIS MELLIFERA* LINNAEUS COLONIES
SELECTED FOR HYGIENIC BEHAVIOUR USING
MICROSATELLITE MARKERS**

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ABSTRACT

In the present study the hygienic behaviour of 10 *Apis mellifera* colonies (7 hygienic: 1H-7H and 3 non-hygienic: 1NH-3NH) was assessed by pin killed brood method and the data on mean percentage of brood removal was recorded after 24, 48 and 72 h. It was observed that after 24 h, the mean dead brood removal was 88.93 and 50.11 per cent in hygienic and non-hygienic colonies, respectively. 40 microsatellite markers including the five markers associated with the hygienic behaviour QTLs were used to determine the molecular variability of the colonies. The 5 microsatellite loci did not differentiate the hygienic and non-hygienic into two distinct clusters. Among the 40 markers, 37 were polymorphic. These 37 markers produced a total of 137 scorable DNA fragments with an average of 3.7 fragments per marker. Cluster analysis based on these markers divided all genotypes of the 10 colonies into 3 clusters. Cluster I comprised of four hygienic colonies 7H, 6H, 1H and 2H. The colonies 5H, 2NH, 4H, 3H, and 1NH made up the cluster II and the cluster III consisted of a single 3NH colony. These results suggest that the microsatellite markers are efficient for molecular variation studies. Selection of colonies based on the genotypes of individuals and selective breeding programs can enhance the levels of hygienic response and provide commercially significant increase in resistance to diseases and parasites.

Keywords: *Apis mellifera*, hygienic behaviour, microsatellite markers, molecular variability

Introduction

The honey bee, *Apis mellifera* Linnaeus originated in eastern tropical Africa, spread to northern Europe and east into India and Tien Shan range in China due to evolutionary forces governed by natural and artificial selection (The Honey Bee Genome Sequencing Consortium 2006). Presently, honey bees live in every part of the world except the North Pole and the South Pole. They are important not only for the honey they produce; but are also the most dominant pollen vectors of agricultural and horticultural crops. It has been estimated that one-third of the human diet can be traced directly or indirectly to bee pollination (Delaplane and Mayer, 2000) and these also play an important ecological role in conserving the biodiversity (Brown and Paxton, 2009). A honey bee colony is characterized by high genetic diversity among its workers, generated by high levels of multiple mating by its queen. Polyandry generates honey bee colonies with high genetic similarity among workers within a patriline, and genetic variability among workers of different patrilines. For any trait with a genetic component, worker phenotypes within a patriline should be more similar to each other than to worker phenotypes from different patrilines (Oldroyd and Fewell, 2007). Genetic diversity within honeybee colonies has been shown to increase colony fitness (Jones et al., 2004; Mattila and Seeley 2007; Oldroyd and Fewell 2007; Mattila et al., 2008), and influences the level of hygienic behaviour and the

efficiency at which the individual hygienic components are performed (Arathi and Spivak, 2001). Hygienic behaviour is a behavioural response of honeybee workers to infection of brood within their colony. It has evolved as a general mechanism of resistance to brood diseases, and provides significant resistance to a number of commercially important pathogens including *Ascosphaera apis* (the causative agent of chalkbrood) (Gilliam et al., 1983), *Paenibacillus larvae* (American Foulbrood) (Spivak and Reuter 1998a, 2001) and Varroa mites (Harbo and Harris, 1999). It consists of a worker detecting the dead or infected larva or pupa, followed by ‘uncapping’ the wax cover and the removal of the diseased individual (Rothenbuhler, 1964a, 1964b; Arathi, et. al., 2000). A colony has a hygienic phenotype when over 50 per cent of bees are hygienic and an increased percentage of these bees in the colony resulted in a higher efficiency of behaviour performance (Arathi and Spivak 2001; Palacio et al., 2010). Moreover, hygienic bees can modulate the extent of the performance of the behaviour depending on the genetic composition of the colony (Arathi and Spivak, 2001; Borsuk, 2009). The response threshold model of task allocation (Bonabeau et al., 1998; Beshers and Fewell, 2001) suggests that partitioning of hygienic tasks is the result of differing genetic propensities for performing each of the tasks in each worker. Thus, colony genetic architecture is an important level of social organization in honey bees (Calderone and Page, 1988).

Honey bees and bumble bees were the first social insects for which DNA microsatellites were developed (Estoup et al., 1993). A valuable source for loci in honey bees was published by Solignac et al., (2003), including information on subspecies variation and possible amplification in three other species of *Apis mellifera*. For each microsatellite locus, there is a specific set of primers is used for amplification with PCR. Microsatellites have shown to be the most popular genetic marker as the high degree of polymorphism is ideal to study small geographical scales of species. DNA microsatellites offer variation useful for inferring population differentiation, originating from a high mutation rate (Levinson and Gutman, 1987) during replication, by adding or removing repeat units compared to the original microsatellite element. Additionally, microsatellite markers have been reported for honey bees (Estoup et al., 1993, 1994, 1995; Hall, 1991; Oldroyd et al., 1994; Rowe et al., 1997; Solignac et al., 2004) and have been used along with mtDNA to study genetic structure of honey bee populations (Franck et al., 2001; Susnik et al., 2004; de La Rua et al., 2003, 2006) although they show discordant patterns. Microsatellites are tandem repeats of short

units of DNA (1-5bp) that occur with high frequency throughout the genomes of many organisms (Estoup et al., 1993, 1994; Rowe et al., 1997; Solignac et al., 2004). They are polymorphic in nature and have an advantage of selective neutrality (Estoup et al., 1995; Rowe et al., 1997; Solignac et al., 2004). The present study was conducted to determine the molecular variability of *A. mellifera* colonies selected for hygienic behaviour using microsatellite markers as colony level diversity is an essential feature to maintain the colony health and fitness.

Materials and methods

Collection of honey bee specimens

The 100 *A. mellifera* colonies headed by naturally mated queens at *A. mellifera* Apiary, Entomological Research Farm, Department of Entomology, Punjab Agricultural University, Ludhiana were assessed for hygienic behaviour by pin-killed brood method (Newton and Ostasiewski, 1986) for 3 alternate brood cycles at 6 weeks interval between each of the two consecutive test brood cycles; and seven most hygienic and three most non-hygienic colonies were selected from these screened out colonies. The 7 hygienic colonies selected were designated as 1H-7H and the non-hygienic colonies as 1NH-3NH. These 10 colonies were again assessed for hygienic behaviour as above. The percentage of pin killed brood cells removed and brood cells only uncapped in each colony was recorded after 24, 48 and 72 h. 10 adult worker bees were collected from each colony for 3 alternate brood cycles i.e. at 6 weeks interval between each of the two consecutive test brood cycles. Thus from each colony, 30 samples were collected and in total 300 adult worker honey bees were collected from the ten selected colonies (210 samples from 7 hygienic colonies and 90 samples from 3 non-hygienic colonies) representing the three test brood cycles. Specimens were preserved in 100 per cent ethanol and kept at -20°C till DNA isolation.

Total DNA extraction, PCR conditions and Gel electrophoresis

Total DNA were isolated from the entire bee using standardized CTAB method (Evans et al., 2013) using 600 µl of extracting buffer per bee. The DNA isolated was quantified using Thermo Scientific NanoDrop™. All the 300 individual specimens were genotyped at 40 microsatellite loci (Solignac et al., 2007) distributed across the 2, 5, 9, 10 and 16 linkage groups. Among these 40 markers, the microsatellite loci for the QTLs *Hyg1*, *Hyg2*, *Uncap1*, *Uncap2* and *Rem2* reported to be linked with hygienic behaviour, (Oxley et. al., 2010) were also included. The polymerase chain reaction (PCR) assay mixtures (10 µl total volume) consisted of 2.0 µl of 1X PCR buffer, 0.6-0.8 µl of 1.5mM MgCl₂, 1.5µl of 1mM dNTPs, 0.75 µl of each of forward and reverse primer (5 µM), 0.25 µl of Taq DNA polymerase (5 units/µl), 3.0 µl (60ng) of DNA and distilled de-ionized water. Amplification reactions were performed in 384 wells thermal cycler (Applied Biotech). The PCR programme consisted of initial denaturation at 94°C for 4 min and subsequent for 35 cycles denaturation at 94°C for 1 min, primer annealing at 48-60°C (varied with the individual SSR primer) for 1 min and primer extension at 72°C for 1min. Final extension step was performed at 72°C for 7 min. The PCR products were resolved on 3.0 to 3.5 per cent agarose gel along with standard 50bp DNA ladder (Takara) as DNA fragment size marker. The agarose gel was run at a constant voltage of 120V at 4v/cm for 3 h in 0.5X TBE buffer and stained with Good View. Only the robust and repeatable bands were considered in this study. Gels were visualized under UV light and documented using 'Gel Documentation System.'

Statistical analysis

The data on hygienic behaviour was subjected to analysis of variance (ANOVA) for finding the significance of difference among the colonies w.r.t. their hygienic trait. The PCR-generated SSR band profiles were scored as presence (1) or absence (0), and obtained data were compiled into a binary matrix. Polymorphism levels (percentage of polymorphic loci) were expressed as the percentage of the number of polymorphic loci against all loci analysed.

The data matrix was computed with DARwin version 6.0 to produce genetic distance matrix (Perrier and Jacquemond-Collet, 2006). Computed statistics data of genetic variation for all loci are: Polymorphic information content (PIC) value for each polymorphic SSR marker was calculated according to PIC value = $2f_i(1-f_i)$, where, f_i is frequency of amplified allele and $(1-f_i)$ is frequency of null allele. Diversity index/ Genetic diversity is the expected heterozygosity and was calculated according to Weir (1996) as: $DI = 1 - 1/L \sum_i p_i^2$, where p_i is the frequency if i th allele at 1 locus and L is the number of loci. Marker index for SSR markers calculated to characterize the capacity of each primer to detect polymorphism loci among the genotypes. It is the product of DI and EMR (Effective Multiplex Ratio) according to Powell *et al* (1996). Resolving power (Rp) of primers was calculated according to Prevost and Wilkinson (1999): $R_p = \sum IB$, where IB (band informativeness) is equal to $1 - [2 \times (0.5 - p)]$, p is the proportion of genotypes containing the band. Genetic relationships among individual honey bee specimens from the 7 hygienic and 3 non-hygienic colonies were visualized in a dendrogram constructed by unrooted neighbour joining tree using DARwin version 6.0 software.

RESULTS

Evaluation of hygienic behaviour

After 24 h the mean of dead brood cells emptied was 88.93 per cent (range: 85.11-93.33%) in 7 hygienic colonies and 50.11 per cent (range: 42.67-56.67%) in non-hygienic colonies. After 48 h, the mean pricked brood removed was 93.28 per cent (range: 91.78-95.11%) in the 7 hygienic colonies and 72.89 per cent (range: 63.56 to 78.33%) in the non-hygienic colonies. As compared to data recorded after 48 h, no significant difference in the mean percentage removal was observed in the hygienic colonies after 72 h, while two of the non-hygienic colonies reached the hygienic level (Table 1). The mean of dead brood cells only uncapped was 2.85 per cent (range: 1.33-8.11%) per cent in 7 hygienic colonies and 23.33 per cent (range: 20.89- 25.67%) in the 3 non-hygienic colonies. The data was recorded for three days, for the non-hygienic colonies were slow in the act of removing the pricked brood during the same time period when the hygienic colonies have reached the maximum level of removal of brood cells.

Microsatellite loci analysis

DNA fragments were amplified with the selected microsatellite primers. Based on the banding profiles and the polymorphism produced by the 5 SSR loci reported to be linked with hygienic behaviour; [K0263, A058 and K1601 the nearest markers identified for the QTL *Hyg1*,

Hyg2 and *Hyg 3*, respectively; AT128 and K1601 the nearest markers for the suggestive QTL responsible for uncapping component (*Uncap1* and *Uncap2*) and AC074 for the removing component (*Rem1*)], it was observed that these markers did not discriminate between the hygienic and non-hygienic colonies and all the five markers showed polymorphism.. The allelic richness (average number of alleles per locus) of markers K0263, A058, K1601, AT128 and AC074 ranged from 2-4 and all the markers were 100 per cent polymorphic. The diversity index, resolving power, marker index and PIC of all the five markers ranged between 0.37-0.80, 2.39-6.15, 0.83-2.41 and 0.23-0.60, respectively. Of all the 40 microsatellite loci genotypes, 37 were polymorphic and 3 loci were monomorphic. These 37 markers produced a total of 137 scorable DNA fragments with an average of 3.7 fragments per marker of which 134 (97.81%) were polymorphic and 3 monomorphic (2.19%). The number of loci per marker ranged between two in markers UN368, A058, AC109 and K1610 and seven in markers SV198 and 5983, respectively, while three markers produced six alleles each, one marker five alleles, 11 markers amplified four alleles each, 16 markers produced three alleles each. The size of the amplicons ranged between 80-450bp (Table 3).

Polymorphic information content

The PIC values analyzed (Table 4) to characterize the capacity of each SSR markers to show polymorphic loci, ranged between 0.23 (K0263) to 0.77 (marker 5983). The average PIC value for all the evaluated loci was 0.52. Diversity index (DI)/ genetic diversity is the expected heterozygosity. The maximum diversity index was 0.92 for the marker SV198 and minimum of 0.37 was observed for the marker K0263. The mean DI value for all the evaluated markers was 0.68. Marker index (MI) is the capacity of each primer to detect polymorphism. The marker SV198 also recorded maximum index value and minimum was seen for the marker UN368 and the average MI value was 2.56. The Resolving power (Rp) ranged between 2.25(B1293) and 6.30 (AP094) with a mean value of 3.4. Except for the three markers (UN368, K1610 and SV026) all the markers showed 100% polymorphism.

Genetic Diversity

The dendrogram (Fig. 1) depicting the genetic diversity between the colonies and relationship based on the SSR scoring data using weighted Neighbour Joining method was produced by the DARwin software version 6.0. The clustering pattern of the Neighbour joining dendrogram representing the 1H-7H hygienic colonies and 1NH- 3NH non-hygienic colonies comprised of three clusters. Cluster I comprised of four hygienic colonies 7H, 6H, 1H and 2H. The colonies 5H, 2NH, 4H, 3H, and 1NH made up the cluster II and the cluster III consisted of a single non-hygienic 3NH colony. The similarity coefficient based on genomic DNA amplification of 300 samples of honey bee workers using microsatellite markers was estimated by dice similarity coefficient. The similarity coefficient, for the colonies across all the three test brood cycles ranged between 0.47-.98 with a mean value of 0.72.

Discussion

The hygienic behaviour of the 10 colonies was assessed by pin killed brood method and was observed that after 24 h the mean percentage of dead brood removed was 88.93 per cent in hygienic colonies and 50.11 per cent in non-hygienic colonies. After 48 h, in the 7 hygienic colonies, mean brood removal was 93.28 per cent and 72.89 per cent in the non-hygienic colonies. Thus, it was observed that time needed to detect and remove brood in the two groups was variable *i.e.*, hygienic colonies differentiated and removed deadbrood faster than non-hygienic colonies in the same period. The mean percentage of dead brood cells only uncapped was 2.85 per cent in 7 hygienic colonies and 23.33 per cent in the 3 non-hygienic colonies. It could be inferred that the hygienic colonies had fewer hygienic bee performing uncapping activities than the non-hygienic colonies and this observation is in line with the findings of Scannapieco, et. al., (2016). These findings also support the earlier findings of Palacio (2000) who evaluated the colonies for hygienic behaviour using a pin-killed brood assay and reported that total hygienic behaviour in the population, headed by queens without mating control was 84.56 per cent. Bigio et al., 2014, also observed that colonies headed by naturally-mated queens had high levels of hygiene. Al-Ghamdi (2002) found that *A.m.jemenitica* removed cent per cent of dead brood through 24 h, while *A.m. carnica* removed only 55 per cent in the same period. Honey bee colonies show variability in the expression of this behaviour (Perez-Sato et al., 2009; Bigio et al., 2014). Depending on the genetic composition of the colony (Arathi and Spivak, 2001; Borsuk, 2009) hygienic bees can modulate the extent of the performance of the behaviour. Individual patterns of task performance are affected by the individual bee genotype and by the genotype of the nest mates in the colony. Thus, the combined and interactive effects of individual bees with diverse genetic propensities to detect and remove diseased brood affect the colony-level expression of hygienic behaviour (Perez-Santo et al., 2009).

Variation in repeat numbers, an important characteristic of microsatellites, has been exploited extensively as genetic markers, and most of the microsatellite databases offer specific marker information of microsatellite loci. Microsatellites are abundant in the genome of the honeybee and have been used to create a linkage map of *A. mellifera* L. (Solignac et al. 2004) and have long been used for genetic analyses of bees (Estoup et al. 1993; 1995; Oldroyd et al., 1994). The application of microsatellite markers has been done in numerous studies for examining population density and decline (Paini 2004), conservation genetics, (Haig 1998; Solignac et al., 2003), introduction of foreign subspecies (Bodur et al., 2007), as well as for revealing relationships among individuals, population genetic structure analysis (Shaibi et al., 2008); the present study of assessing the molecular variability of *A.mellifera* colonies selected for hygienic behaviour using microsatellite loci is the first of its kind conducted. Through the present study, the utility of selected microsatellite markers for conducting the genetic diversity studies within and between the individuals of the population has been highlighted. The clustering pattern

of the Neighbour joining dendrogram representing the 1H-7H hygienic colonies and 1NH- 3NH non-hygienic were distinguished into three clusters. Cluster I comprised of four hygienic colonies 7H, 6H, 1H and 2H. The cluster II comprised of colonies 5H, 2NH, 4H, 3H, and 1NH and the cluster III consisted of a single non-hygienic 3NH colony. The majority of the individuals of each colony showed no overlapping with individuals from different colonies except for few individuals. The individuals from colonies 6H intermingled with those of 7H. The individuals of colony 2NH formed group with 5H and 3H whereas individuals of colony 3H grouped with 1 and 3 NH. The present findings are in corroboration of the studies conducted by Munoz, et al., (2008) where three types of honey bee pupae: pupae not parasitized, infested pupae with mites without reproductive success and infested pupae with mites with reproductive success were genotyped with five microsatellite *loci* and microsatellite analysis revealed that different groups of pupae can be also distinguished by a different allelic composition. In the principal coordinate analysis, two main clusters of individuals were distinguished. Further selective breeding programs can enhance the levels of hygienic and provide significant resistance to diseases and parasites. Colony level genetic diversity plays a major role in both disease resistance and division of labor, resulting in greater productivity and colony strength.

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Table 1. Mean percentage of brood cells removed after 24, 48 and 72h of pricking

Colony No.	24h	48h	72h
1H	85.11 (67.47)	91.78 (73.50)	93.00 (74.70)
2H	93.33 (75.17)	94.56 (76.67)	94.56 (76.67)
3H	92.22 (73.84)	93.78 (75.68)	94.11 (76.06)
4H	92.56 (74.55)	95.11 (77.80)	95.67 (78.37)
5H	85.44 (67.80)	91.78 (73.44)	93.11 (74.89)
6.H	88.11 (69.96)	92.44 (74.75)	93.67 (75.92)
7H	85.78 (68.10)	93.56 (75.51)	95.33 (77.56)
1NH	42.67 (40.76)	63.56 (52.85)	75.89 (60.63)
2NH	51.00 (45.55)	76.78 (61.29)	84.33 (66.86)
3NH	56.67 (48.84)	78.33 (62.28)	83.78 (66.31)
LSD (p=0.05)	(1.88)	(2.03)	(1.57)

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

Table 2. Percentage of brood cells only uncapped after 24, 48 and 72h of pricking

Colony No.	24h	48h	72h
1H	5.22 (12.78)	1.11 (3.99)	0.44 (3.99)
2H	1.33 (4.37)	0.44 (1.28)	0.44 (1.28)
3H	1.44 (4.57)	0.89 (3.98)	0.56 (3.98)
4H	2.00 (6.58)	1.33 (4.86)	0.78 (4.86)
5H	6.33 (11.47)	1.22 (3.67)	0.22 (3.67)
6.H	2.22 (6.60)	1.89 (6.41)	0.89 (6.41)
7H	8.11 (15.98)	2.11 (6.77)	0.33 (6.77)
1NH	25.67 (30.08)	12.89 (20.58)	2.89 (20.58)
2NH	23.44 (28.89)	3.78 (9.79)	1.22 (9.79)
3NH	20.89 (27.16)	3.56 (10.53)	1.56 (10.53)
LSD (p=0.05)	(2.27)	(3.44)	(3.13)

Data values represent mean of 3 replications

Figures in parentheses are the means of arc sine $\sqrt{\text{percentage}}$ transformation

Table 3. Summary of PCR amplification of 37 SSR markers used for genetic analysis of the hygienic and non -hygienic colonies

S.No.	Name of the Marker	Total no. of Loci	Monomorphic Loci	Polymorphic Loci	Size Range(bp)	Polymorphic %
1.	K0206	5	0	5	110-155	100
2.	K0264	4	0	4	200-230	100
3.	AP098	4	0	4	100-135	100
4.	K0263	4	0	4	290-310	100
5.	UN368	2	1	1	240-250	50
6.	SV198	7	0	7	100-450	100
7.	BI036	3	0	3	135-160	100
8.	SV172	3	0	3	130-150	100
9.	A058	2	0	2	80-85	100
10.	K0519	6	0	6	160-200	100
11.	5983	7	0	7	102-150	100
12.	K0520	4	0	4	190-220	100
13.	K0521	6	0	6	224-270	100
14.	AC011	3	0	3	127-150	100
15.	AT128	4	0	4	186-210	100
16.	BI293	3	0	3	182-200	100
17.	SV026	3	1	2	166-170	66.67
18.	AG016	3	0	3	220-240	100
19.	K0902	4	0	4	156-200	100
20.	5242	4	0	4	316-340	100
21.	K1076	4	0	4	200-235	100
22.	K1074	4	0	4	201-230	100
23.	AC074	3	0	3	189-200	100
24.	AP146	4	0	4	151-170	100
25.	AC109	2	0	2	119-125	100
26.	AC138	4	0	4	164-180	100
27.	BI119	3	0	3	172-180	100
28.	K1012	3	0	3	163-175	100
29.	BI324	3	0	3	125-140	100
30.	K1601	3	0	3	177-190	100
31.	AP217	3	0	3	185-200	100
32.	AP028	3	0	3	230-240	100
33.	K1610	2	1	1	265-270	50
34.	AP094	6	0	6	133-160	100
35.	K1638	3	0	3	198-210	100
36.	6560	3	0	3	166-180	100
37.	K1656	3	0	3	231-240	100

Table 4. Diversity index, Resolving power, Marker index and PIC values of SSR primers

Marker	Diversity Index (DI)	Resolving power (Rp)	Marker Index (MI)	Polymorphism information content (PIC)
K0206	0.50	6.17	2.50	0.38
K0264	0.80	2.45	3.20	0.69
AP098	0.71	3.70	2.83	0.54
K0263	0.37	6.15	0.83	0.23
UN368	0.48	2.37	0.24	0.41
SV198	0.92	3.58	6.41	0.74
BI036	0.52	3.67	1.55	0.39
SV172	0.69	3.28	2.08	0.45
A058	0.61	2.48	1.21	0.38
K0519	0.83	3.83	4.96	0.68
5983	0.85	3.29	5.93	0.77
K0520	0.69	3.86	2.75	0.52
K0521	0.74	4.87	4.41	0.59
AC011	0.72	2.27	2.17	0.62
AT128	0.78	3.58	3.13	0.55
BI293	0.74	2.25	2.21	0.57
SV026	0.65	2.64	1.94	0.56
AG016	0.72	2.86	2.15	0.52
K0902	0.74	3.31	2.96	0.59
5242	0.66	4.27	2.63	0.47
K1076	0.81	3.41	3.24	0.57
K1074	0.74	3.91	2.97	0.51
AC074	0.80	2.47	2.41	0.59
AP146	0.84	2.78	3.37	0.65
AC109	0.45	2.88	0.90	0.28
AC138	0.73	3.95	2.94	0.51
BI119	0.56	3.61	1.69	0.40
K1012	0.72	2.62	2.17	0.56
BI324	0.68	2.80	2.03	0.53
K1601	0.68	2.39	2.05	0.60
AP217	0.72	2.99	2.17	0.50
AP028	0.74	3.08	2.21	0.49
K1610	0.47	2.51	0.95	0.37
AP094	0.57	6.30	3.43	0.55
K1638	0.65	3.09	1.94	0.49
6560	0.70	3.01	2.09	0.50
K1656	0.73	3.09	2.20	0.48
Mean	0.68	3.4	2.56	0.52

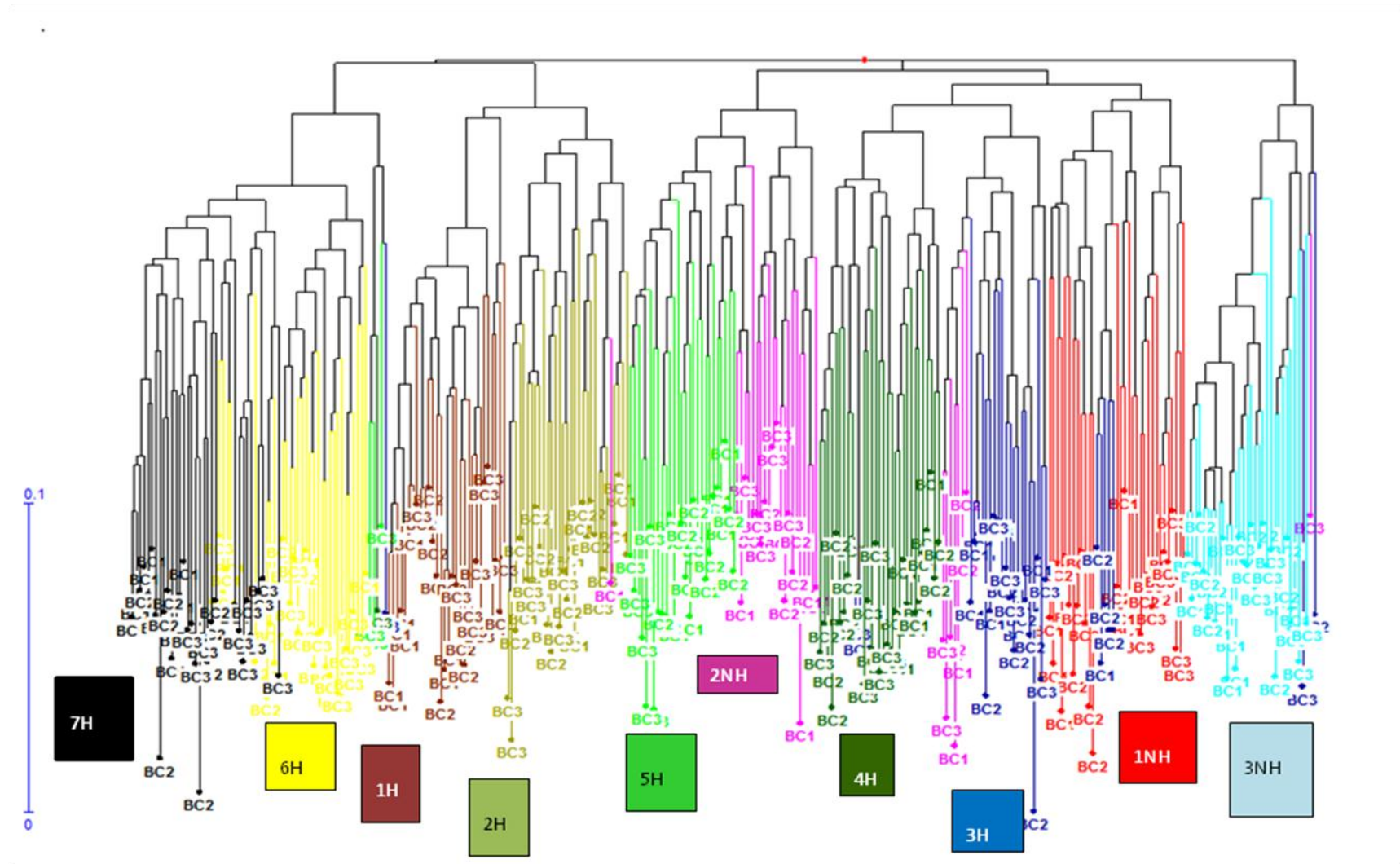


Figure 1. Neighbour joining dendrogram showing clustering pattern of 1H-7H hygienic colonies and 1NH- 3NH non-hygienic colonies in all the three alternate brood cycles

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