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Selection of Larvae for Queen Rearing by Workers in the Honey Bee (*Apis mellifera* L.) − *Nepotism or Different Maternal Supply*?

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von
AL-Kahtani, Saad Naser Saad

Präsident der Humboldt-Universität zu Berlin
Prof. Dr. Jan-Hendrik Olbertz

Dekan der Landwirtschaftlich-Gärtnerischen Fakultät
Prof. Dr. Dr. h. c. Frank Ellmer

Gutachter: 1. Prof. Dr. agr. habil. Kaspar Bienefeld

2. Prof. Dr. agr. Kurt-Johannes Peters

3. PD Dr. Ricarda Scheiner

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Abstract

Natural selection favours an efficient cooperation within eusocial colonies. However, in polyandrous species, queen rearing may provide some conflict. Worker bees are assumed to play a nepotistic role during the queen-rearing phase by preferring more closely related larvae. Honeybee workers were found to be able to discriminate between different related individuals, but published data are inconsistent. Here in my study I show that larvae reared on a standardized basis were not significantly preferred by related worker bees, but that a higher egg weight – from which the larvae originated – significantly increased the chance to receive a royalty treatment and showed a tendency to correlate positively with fitness traits of the resulting queen. Queen rearing results from a colony decision, and this involves many workers. Consequently any lack of a nepotism which is proved may be due to the fact that relatedness-driven kin preference of individuals cannot be applied to a colony decision. In view of this, using DNA fingerprinting, I also tested the relatedness of larvae to be reared as queens and those nursing worker bees which initiated the queen−rearing process. In five colonies, each inseminated with three drones from three different origins, I did not observe any preference of worker bees for their closely related sib for queen rearing, but I did find significant differences with respect to the preference of worker bees for larvae from the three drone origins. Our data provide evidence that honey bees have the ability to discriminate genetic differences, but they do not use it for nepotism. In a natural, diverse – but always at least 25%-related – framework within a colony, genetically or environmentally driven attractiveness but not relatedness represents the key criteria for selecting larvae for gyne production.

Keywords:

Egg weight, Inclusive fitness, Nepotism, Queen rearing

Zusammenfassung

Natürliche Selektion bevorzugt effiziente Zusammenarbeit innerhalb von eusozialen Kolonien. Dennoch kann bei polyandrischen Arten die Aufzucht von Königinnen Konflikte auslösen. Es wird vermutet, dass Honigbienen nepotistisch bei der Königinnenaufzucht agieren. Bienen sind in der Lage zwischen verschiedenen verwandten Individuen zu unterscheiden. Die bisher publizierten Daten sind aber widersprüchlich. In meiner Untersuchung zeigte sich, dass standardisiert aufgezogene Larven durch verwandte Arbeitsbienen nicht signifikant bevorzugt werden, aber dass ein höheres Gewicht der Eier, aus denen die Larven geschlüpft sind, ihre Chancen als Königin aufgezogen zu werden signifikant erhöhen. Ein hohes Eigewicht führte außerdem zu einer zwar nicht signifikanten, aber tendenziellen Erhöhung von Fitness Eigenschaften der resultierenden Königinnen. Probleme, Nepotismus eindeutig zu belegen, sind möglicherweise dadurch verursacht, dass die Verwandtschafts-Präferenz bestimmter Individuen nicht in eine Kolonie-Entscheidung überführt werden kann. Ich habe deshalb – mit molekulargenetischen Methoden – die Verwandtschaft der Larven, die als Königinnen aufgezogen werden sollen, mit jener der Pflege-Bienen, die deren Aufzucht initiierten, vergliechen. In fünf Kolonien, deren Königinnen mit jeweils drei Drohnen von drei verschiedenen Herkünften besamt wurden, fand ich keine Präferenz von Arbeitsbienen für ihre nah-verwandten Geschwister. Dafür zeigten sich signifikante Unterschiede bezüglich Präferenz für Larven der drei Drohnen-Herkünfte. Dies kann so interpretiert werden, dass Honigbienen in der Lage sind, zwischen genetischen Varianten zu unterscheiden, aber dass diese Fähigkeit nicht für Nepotismus genutzt wird. In einem natürlichen, durch Mehrfachpaarung genetisch variablen, aber mindestens zu 25 % verwandten Volk, ist eine genetisch oder umwelt- (maternal) induzierte Attraktivität, und nicht der Grad der Verwandtschaft, das zentrale Selektionskriterium in.

Schlagwörter:

Gewicht der Eier, Fitness Eigenschaften, Nepotismus, Aufzucht von Königinnen

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1 General introduction

Few organisms are as well studied as the honey bee (*Apis mellifera* L.). It is the sole subject of research in several university departments and research centres and has several journals devoted specifically to it. This is partly attributable to its economic importance in agricultural systems, but also because of its unique biology and relative ease of access and manipulation for study.

The evolution of co-operation in eusocial insects has for decades been a central topic in evolutionary biology. The key trait of eusocial ("truly social") species is that most of the colony members (workers) give up their own chances of reproduction and help raise the offspring of nestmates, which are highly fecund (sexuals or reproductives).

Eusociality according to Wilson (1971) is defined by the following three characteristics: 1) the partition of reproduction among the colony members, with sterile or subfertile workers and highly fecund sexuals or reproductives, 2) overlapping adult generations and 3) co-operative brood care. All ants and termites, some bees and wasps, ambrosia beetles (Kent and Simpson, 1992), aphids (Aoki, 1987; Benton and Foster, 1992), thrips (Crespi, 1992), shrimps (Duffy, 1996) and some naked mole-rats (Sherman *et al*., 1991; Jarvis *et al*., 1994) have been found to fit this definition.

In eusocial insects, for instance, we have to consider that differences in relatedness within colonies of social Hymenoptera are likely to create a variety of conflicts and cooperation between colony members. Furthermore, kin selection has been widely accepted by the scientific community. Insect societies have long served as a useful model with regard to these issues in evolutionary biology, i.e. the level at which natural selection operates.

Kin recognition can be defined as the ability of an individual to distinguish kin from non-kin or to differentiate between different classes of kin (Hepper, 1986). Such recognition appears to be common in the animal kingdom (Hepper, 1991), by using a widely involved mechanism to recognize kin not previously encountered, and has been demonstrated in monkeys (Wu *et al*., 1980), a social spider (Evans, 1999), a sweat bee, (Greenberg, 1979), ants (Jutsum *et al*., 1979; Mintzer, 1982; Lenoir, 1984), paper wasps (Allen *et al*., 1982; Pfennig *et al*., 1983; Hepper, 1986; Gamboa, 2004) and the honey bee (Breed, 1981 and 1983; Page and Erickson, 1984; Getz and Smith, 1986; Page *et al*., 1989; Carlin and Frumhoff, 1990). Additionally, among mammals, most work on kin recognition has involved rodents: mice, rats, squirrels and voles (Gadagkar, 1985).

Kin selection can also explain the evolution of sociality and cooperation among individuals. Inclusive fitness theory, for the first time, provides a framework for explaining the way that the level of relatedness between individuals can compensate the costs associated with altruism and can influence the occurrence of social behaviour (Hamilton, 1964).

Hamilton (1963; 1964a, b) was the first to develop kin selection and viewed it as a far-reaching important evolutionary principle. His “kin selection theory” states that individuals can transmit copies of their genes not only directly through their own reproduction, but also indirectly, by favouring the reproduction of kin. Altruistic behaviour should be favoured if the ratio of the costs (c) accruing to the donor of the altruistic act to the benefits (b) gained by the beneficiary is lower than the relatedness (r) of the recipient to the donor of the altruistic behaviour (Hamilton’s rule, $c/b < r$). Here, benefit means the enhanced production or survival of offspring by the beneficiary, whereas cost means the number of offspring lost by the altruist.

Kin selection theory also implies the occurrence of potential kin conflicts, because, in contrast to the cells of an organism, nestmates are not genetically identical (Ratnieks and Reeve, 1992; Keller and Reeve, 1999). Hence, kin selection predicts a dynamic equilibrium between co-operation and conflict, depending on, for example, the genetic composition and size of a colony, the benefits and costs of group membership, and the benefits and costs of selfish behaviour and policing (Keller and Chapuisat, 1999).

Social insect species, for example, are particularly interesting models to study altruism, because the haplodiploidy that characterizes Hymenoptera, the order of ants, bees and wasps, can lead to high levels of relatedness between individuals. However, insect colonies show a great variability in their social organization and this results in highly variable levels of relatedness among colony members (Zinck *et al*., 2009). However, recently, Nowak *et al*., (2010) have speculated that higher relatedness is not necessarily the best option for inclusive fitness and that, therefore, relatedness is irrelevant for eusociality.

Polyandry (females mating with several males) occurs commonly and is a widespread phenomenon in social Hymenoptera (Page, 1986; Ross, 1986; Have *et al*., 1988; Arnqvist and Nilsson, 2000; Jennions and Petrie, 2000; Crozier and Fjerdingstad, 2001; Maklakov and Lubin, 2006); this has important consequences for reproductive conflict and cooperation among colony members. For a honey bee, the colony consists of a single queen, tens of thousands of sterile female workers and usually a few hundred drones (Winston, 1987). The honey bee (*A. mellifera*) is a particularly important model organism in studies of social cooperation and conflict, because of the genetic variation within a colony (Oldroyd and Fewell, 2007). In general, colony honey bees normally comprise 10-20 patrilines (Estoup *et al*., 1994; Robinson *et al*., 1994; Arnold *et al*., 1996; Oldroyd *et al*., 1997). Workers within the

same patriline share an average coefficient of relatedness of 0.75 (full-sister) and among patrilines 0.25 (half-sister) (Ratnieks and Reeve, 1991; Châline *et al*., 2003; Châline and Arnold, 2005). Furthermore, *A. mellifera* workers are able to discriminate the degree of relatedness to themselves of larvae (Visscher, 1986; Noonan, 1986). Because of relatedness differences, workers can potentially gain a threefold increase in inclusive fitness if they can induce a full-sister instead of a half-sister to head a new colony (Visscher, 1998). Occasionally, this involves preferentially rearing queens from related larvae (Breed, 1983; Page and Erickson, 1984; Page *et al*., 1989; Carlin and Frumhoff, 1990). For this, worker bees possess the sensory capabilities and behavioural responses that would enable them to maximize their individual inclusive fitness through nepotism in queen rearing. This is an important aspect in kin selection theory. However, several studies with regard to *A. mellifera* have been unable to establish nepotism in queen rearing. Less related larvae exhibit a similar or even higher acceptance for queen rearing than do larvae with a higher degree of relatedness (Woyciechowski, 1990; Breed *et al*., 1994).

Hence, queens' production in these societies involves numerous group decisions with respect to foraging, nest maintenance and reproduction. This is because the inclusive fitness of the whole colony depends strongly on decisions made during this process (Tarpy and Gilley, 2004). Therefore, colonies show a variety of complex behaviours that far transcend those of the individual colony member and which cannot be accounted for by any apparent central control or simple hierarchical structure (Hölldobler and Wilson, 1990).

The purpose of this work has been to focus on whether the acceptance of larvae for queen rearing is also affected by any factor over and above genetic relatedness. Only a few studies have shown a significant preference for related larvae (Page and Erickson, 1984; Noonan, 1986; Visscher, 1986; Tarpy and Fletcher,

1998; Koeniger *et al*., 1996; Mohammedi and Le Conte, 2000). However, all available studies have revealed a significant preference towards related larvae. Nevertheless, most of these studies involved larvae which had hatched in non-related colonies of origin, so that recognition might not have been based on kin recognition, but rather on colony odour, which is partly environmental and can be differentiated by worker bees. Consequently, a special experimental design has been created to exclude any influence of colony odour of larvae to be reared as queens. Consequently, we used age-standardized larvae that had hatched and were reared under standardized conditions outside of their hives.

In this thesis, I first examine and create a methodological basis for the study of differential prenatal maternal investment shown in honey bees, by determining the repeatability of egg weight measurements and by identifying the optimum age of eggs for measuring differences in maternal investment. Then, I speculate that the initial chance of a larva related to the individual weight of the egg from which it originated will affect its later performance. Consequently, rearing queens from larger eggs might help to optimize colony fitness. This is perhaps more important than selection attributable to relatedness to a special subgroup.

Finally, queen rearing results from a colony decision, which involves many workers; consequently the lack of significantly proving nepotism may be due to the fact that relatedness-driven kin preference of individuals cannot be transferred into a colony decision. Therefore, I tested also by DNA fingerprinting the relatedness of larvae to be reared as queens and the nursing worker bee, which initiates the queen rearing process, in order to determine the influence of reaction towards kin.

2 Experimental part of the study

2.1 Studies on the variability of maternal investment in offspring

2.1.1 Introduction

The supply of nutrients to eggs is an investment of the mother into the fitness of her offspring. Studies in species of almost all major taxa of egg-laying animals have shown that the amount of provisions contained in the propagule, usually approximated by measuring its size or weight, can influence the size, vitality or reproductive success of offspring (e.g. Kaplan, 1987; Russell *et al*., 2007). In situations where environmental conditions are stable, oviparous females tend to lay eggs which are all receive similar quantities of nutrients (reviewed in Crean and Marshall, 2009; Marshall *et al*., 2008). If environmental conditions are unpredictable however, theory predicts that mothers should produce both small and big eggs as a way of “hedging their bets”. If conditions are favourable, a great proportion of offspring both from light and heavy eggs can survive. If they are unfavourable, at least some will likely be able to carry on the genes of their parents (Cooper and Kaplan, 1982; Philippi and Seeger, 1989; Crean and Marshall, 2009). In the honeybee (*Apis mellifera*), effects of environmental changes on the success of brood rearing are probably less immediate than in other species, because the colony and its food stores provide a buffer between exterior influences and the immature stages in their cells. Nevertheless, there are good reasons why a bet-hatching strategy could be adaptive in this highly social species. One is that diversity of worker phenotypes provides a security against changing conditions for the colony as a whole. This is thought to be one reason why polyandry is common in highly eusocial insects, because it increases phenotypic variation among workers by increasing genetic

variation (e.g. Waddington *et al*., 2010; Mattila *et al*., 2008; Oldroyd and Fewell, 2007). Differences between workers can also be caused by differences in maternal investment (Borodacheva, 1973), and this could add to the variability resulting from the presence of several patrilines. A second possible role for differential maternal investment could lie in the fact that female eggs can develop into either queens or workers. It has been shown that queen weight and the number of a queen's ovarioles are strongly influenced by the weight of the egg from which they develop (Borodacheva, 1973). Therefore, the production of some heavier eggs for colony reproduction or as a "precautionary measure" for the case of sudden queen loss could be a selective advantage.

A precondition to studying these interesting possibilities is the existence of variability of the size of egg provisions in the honey bee. The aim of this study is to test whether this precondition is fulfilled. Several studies have already dealt with egg weight variability in *Apis mellifera*, but they focussed mostly on inter-colony differences and environmental influences. Taber and Roberts (1963) and Roberts and Taber (1965) have evidenced considerable differences between queens concerning egg weights, and found that these differences were partly heritable. Borodacheva (1973) weighed eggs from 200 queens and found that the heaviest egg was more than 2.5 times as heavy as the lightest one. Król (1996) showed that the weather had no sizeable influence on egg weight, but that negative correlations between egg weight and the number of eggs produced can sometimes be found (see also Jordan, 1961, for this relationship). Woyke (1998) showed that the size and weight of bee eggs change during development, which means that egg age should be standardized as much as possible if differences in maternal investment are to be measured.

To create a methodological basis for the study of differential prenatal maternal investment in the honey bees, we started off by determining the repeatability of egg weight measurements. We also identified the optimum age of eggs for measuring differences in maternal investment. In the context of possible effects of differential maternal investment on properties of the offspring, it was mainly differences between eggs laid by the same queen which were of interest. Accordingly, our principal aim was to measure variability within samples of eggs laid by individual queens within a short time frame. Secondly, we studied weight differences between the eggs laid by the same queens either in spring (when the so-called “summer bees” are produced) or in late summer (when both “summer” and “winter bees” are produced). Our hypothesis was that if quantitative differences in maternal investment play a role in the differentiation into “summer” or “winter bees”, this should lead to greater variability of egg weights in late summer.

2.1.2 Results

Manuscript 1

Variability of prenatal maternal investment in the honey bee (*Apis mellifera*)

Saad Naser AL-Kahtani, Jakob Wegener, Kaspar Bienefeld

(**submitted** for *Journal of Apicultural Research*)

Summary

The unequal distribution of resources among offspring by females can be an important adaptation to changing or unpredictable environments in many taxa. In the honeybee, a potential role for differential maternal investment could lie in the amplification of intra-colony phenotypic variability, which is an important factor in

stress resilience. Differential maternal investment could also influence polyethism, if it led to physiological adaptations that are important for fulfilling different tasks. Here we asked whether egg weight variability in *Apis mellifera* is great enough to justify such hypotheses. We first determined the repeatability of weight measurements, and the optimum stage at which eggs should be sampled in order to minimize imprecision due to unavoidable intra-sample age differences. We then weighed eggs from fourteen colonies, and assessed the relative level of weight variability. We also compared the means and variabilities of eggs produced in spring and late summer, to test the hypothesis that eggs destined to become a mix of summer and winter bees should be more variable than eggs all destined to become summer bees. The results show that the optimum age for sampling eggs is 48 h. No systematic difference was found between spring and summer samples, but sample means from the same queens differed by up to 22%. Weight of eggs laid by the same queen within six hours varied by up to 58%. Egg weight variability was clearly sufficient to expect phenotypic differences at the adult stage. We conclude that a considerable level of differential maternal investment exists in the honey bee. The study of its consequences and evolutionary significance in social species could be an exciting field for future studies.

2.2 Studies on decision making for queen rearing at quantification factors that determine which individual larvae are chosen as gynes

2.2.1 Introduction

Colonies of social insects make numerous group decisions with respect to foraging, nest maintenance and reproduction. Queen production, for example, is of extreme importance because fitness of the colony strongly depends on decisions made during this process (Tarpy and Gilley, 2004). In honey bee (*Apis mellifera*) colonies, new queens are produced during reproductive swarming, supersedure and emergency queen replacement. In the latter case, only a few larvae from the many available are selected by workers to be reared as gynes (Winston, 1987).

Nepotism has been hypothesized as the underlying reason for the selection of individual larvae to be reared as queens (Tarpy *et al*., 2004). However, the mechanism that initiates this group decision is uncertain (Tarpy and Gilley, 2004). In this study, aimed primarily at quantifying the factors that determine which individual larvae are chosen as gynes, we observed that some workers presented their Nasonov glands after inspecting larvae that were offered to queenless colonies in artificial queen cups. The Nasonov pheromone is known to be involved in the outdoor orientation of honey bees (Sladen, 1901). The fundamental decision-making dilemma for groups is to turn individual preferences into a single choice for the group as a whole (Seeley *et al*., 2006). This observed behavior may be essential to this process. Using video records and observations of the initial phase of the queen rearing process, we sought to determine whether exposure of the Nasonov glands is linked to the recruitment of honey bees for individual larvae to be reared as queens.

2.2.2 Results

Manuscript 2

The Nasonov Gland Pheromone is Involved in Recruiting Honey bee Workers for Individual Larvae to be Reared as Queens

Saad Naser AL-Kahtani and Kaspar Bienefeld
(**accepted** for *Journal of Insect Behavior*)

Summary

Colonies of social insects make numerous group decisions, and queen production has a high impact on their fitness. However, the mechanism which initiates selection of individual larvae to be reared as queens remains uncertain. Infra-red video technique and a modified observation hive provide us insight into the behaviour of worker bees at the initial phase of the queen rearing process. Some workers were found to present their Nasonov gland (ENG) after inspecting larvae which were offered to queenless colonies in artificial queen cups. To our knowledge, these are the first data which show an exposure of Nasonov glands within the hives of honey bees. ENG was found exclusively at cells containing young brood (< 48 h) suitable for queen rearing, and a highly significant concentration of ENG was observed at cells later developing into queen cells. This supports the assumption that ENG is not a general trigger for brood care, but that it is involved specifically in campaigning for larvae to be reared as gynes. ENG is able to attract other bees and successfully recruit specific larvae. However, I also found ENG at cells which were not accepted for gyne production. The duration of ENG (assumed indicator of the intensity of recruiting) did not differ significantly at the cell which was accepted or not accepted for queen rearing. It is likely that not every act of recruiting behaviour results in a group decision. The lack of a significant difference between duration of ENG and a

highly significant worker density after recruiting for cells accepted and not accepted for queen rearing after ENG, respectively, provides strong evidence that differences in the intensity of recruiting do not influence the final group decision, but that differences in the attractiveness of the campaigned larvae do. If the decision of the recruiting bee for an individual larva corresponds with the notion of the group, then the larva's superior attractiveness is likely to fine tune the search for it within the pheromonal sphere of the recruiting worker.

2.3 Studies on which affects selection of larvae to be reared as queen rather than genetic relatedness

2.3.1 Introduction

In the honey bee *Apis mellifera*, mating of queens with several haploid males results in societies with large variations in genetic relatedness. Subfamilies originating from the same drone share on the average 75% common alleles, while among members from different subfamilies, only 25%. Workers may benefit in a nepotistic manner by raising super sisters instead of half-sisters as new queens, thereby increasing their inclusive fitness (Tarpy *et al*., 2004). A precondition for selective queen rearing is the ability of workers to recognize kinship, as was demonstrated by Breed (1981) and Getz and Smith (1983), but results of studies to prove the presence of nepotism in queen rearing were inconsistent (Page and Erickson, 1984; Breed, 1981; Koeniger *et al*., 1996; Breed *et al*., 1984; Visscher, 1986; Woyciechowski, 1990). However, by pooling data of all available studies, a significant preference towards related larvae (n = 14148 larvae) was found ($\chi^2 = 21.3$, $P < 0.0001$). But the most common methods in these studies, in which young brood was reciprocally transferred between colonies

are criticised (Tarpy and Fletcher, 1998) because recognition may not be based on kin but rather on colony odour, which is partly environmental and can be differentiated by worker bees (Breed, 1994).

In the current study, we try to avoid this methodological pitfall, by artificial rearing of transferred larvae and we include, beside relatedness, another factor, which may impact the acceptance for queen rearing. Different prenatal maternal investment was found to affect fitness of the resulting offspring (Sinn *et al*., 2008), consequently this differences in maternal supply may provide a suitable selection criterion at this early stage. Prenatal investment was estimated by measuring egg weight at 48 h from which the tested larvae originated from.

2.3.2 Results

Manuscript 3

Strength surpasses relatedness – how honeybees decide which larvae should be reared as new queens

Saad Naser AL-Kahtani and Kaspar Bienefeld

(**In preparation** for publication)

Summary

Natural selection favours efficient cooperation within eusocial colonies. However, in polyandrous species, queen rearing might provide some conflict. Worker bees are assumed to play a nepotistic role during the queen-rearing phase by preferring more closely related larvae (Getz and Smith, 1983). Honeybee workers have been found being able to discriminate between differently related individuals (Page and Erickson, 1984), although the published data are inconsistent (Tarpy *et al*., 2004). Here, we show that standardized reared larvae are not significantly preferred by related worker

bees, but that a higher egg weight from which the larvae originate significantly increases its chances of receiving royal treatment and shows a tendency positively to correlate with the fitness traits of the resulting queen. Queen rearing results from a colony decision involving many workers. Consequently, the lack of significantly establishing nepotism might be because the relatedness-driven kin preference of individuals cannot be transferred into a colony decision. We have therefore also tested, by DNA fingerprinting, the relatedness of the larvae to be reared as queens and the nursing worker bee that initiates the queen rearing process. In five colonies, each inseminated with three drones of three different origins, we have not observed any preference of worker bees for their closely related sibling for queen rearing but we have found a significant preference of workers for larvae of a special drone origin. Our data provide evidence that honeybees have the ability to discriminate genetic differences but that they do not use this skill for nepotism. In the naturally genetically diverse but always (at least 25%) related framework within a colony, genetically or environmentally driven attractivity and not relatedness are the key criteria for selecting larvae for gyne production. With regard to factors such as cost and benefits in Hamilton's equation (1964a,b), the preference for higher relatedness is not necessarily the best option for inclusive fitness and, consequently, our data do not support the new hypothesis of Nowak *et al*., 2010 proposing that relatedness is irrelevant for eusociality.

3 General discussion

Fundamental evolutionary theories, such as those regarding inclusive fitness (Hamilton, 1964; also known as kin selection by Smith and Wynneedwards, 1964), and sex ratio (Fisher, 1930; Trivers and Hare, 1976) make predictions concerning the occurrence and the extent of various conflict situations in colonies of social insects. Specifically, the new hypothesis as proposed by Nowak *et al*., (2010) argues that relatedness is irrelevant to eusociality. In eusocial insects, little evidence of nepotism has been found within colonies, except when obvious cues such as gender of the offspring are correlated with differences in relatedness (Sundstrom *et al*., 1996; Keller, 1997; Queller *et al.*, 2000; Field *et al*., 2006).

The subject has thus gained new impetus from the re-interpretation of the role of relatedness for the development of eusociality, as sparked by the article of Nowak *et al*., (2010) and the sometimes heated discussions which have followed among 140 biologists (Abbot *et al*., 2011; Boomsma *et al*., 2011; Strassmann *et al*., 2011; Ferriere and Michod, 2011; Herre and Wcislo, 2011). Nowak *et al.* begin by asserting that the classical argument for insect eusociality (cooperatively breeding societies in which “castes” of individuals, like the workers in bees, are sterile and help the queen produce offspring) is an argument based on asymmetrical relate*dness*. We find this assertion to be wrong. This failure to *ex*plain eusociality, they claim, is a severe blow to kin-selection theory. However, evidence for nepotism, for instance in honey bee colonies, remains sparse and controversial. As we have shown above for the honey bee, this is, to our knowledge, the first time that the question has been raised as to whether queen rearing is affected by any factor other than genetic relatedness.

In brief, the most common methods in all studies regarding potential conflict in honey bee societies concern queen rearing under circumstances in which young brood is reciprocally transferred between colonies; such methods have been criticised, because recognition might not be based on kin but rather on colony odour, which is partly environmental and can indeed be differentiated by worker bees. In the current work, I have tried to avoid this methodological pitfall by using artificial rearing of transferred larvae. Moreover, I include, in addition to relatedness, another factor which might impact acceptance for queen rearing. The maternal investment strategy entails a trade-off between the size and number of progeny, so that the daughters upon emergence can best perform in their brood production under the seasonally variable environments where they reproduce. In addition, an ample body of literature shows that superior maternal supply positively impacts the fitness of offspring in many taxa (Marshall and Keough, 2008; Moran and McAlister, 2009). In animals, offspring developing from heavier eggs generally grow faster, attain a larger size, and have higher survivorship than progeny developing from lighter eggs (Kaplan, 1991; Reznick, 1991; Fox, 1994). However, mothers laying large eggs must lay fewer eggs because of the trade-off between size and number of offspring (Smith and Fretwell, 1974; Fleming and Gross, 1990; Berrigan, 1991), which results in an egg size that is a balance between selection for larger-sized eggs and selection for a large number of eggs.

Phenotypic variations of honey bees reared simultaneously within a colony have important implications for the functioning of the colony, especially for task-sharing between its members (Kerr and Hebling, 1964; Waddington, 1989; Makert *et al*., 2006). A relationship between egg weight and offspring phenotype has been shown in many species (e.g. Dzialowski and Sotherland, 2004; Bonato *et al*., 2009). Furthermore, progeny fitness usually increases with increasing parental investment

per offspring, thus favouring the production of large-sized progeny (Azevedo *et al*., 1997; Fox and Czesak, 2000). For instance, larger offspring have frequently been found to mature earlier, to have an improved ability to withstand competition, or to survive better within stressful environments when compared with small offspring (Azevedo *et al*., 1997; Fox and Czesak, 2000; Czesak and Fox, 2003; Roff, 2002; Fischer *et al*., 2003; Fischer *et al*., 2006).

In the honey bee, for instance, Bilash *et al*., (1985) have compared morphological traits of workers reared within the same colony. Moreover, Król (1996) has studied the variability of egg weight in five colonies headed by sister queens. She found ranges that are rarely below 20%, and these reach up to 66% of the respective means in samples produced by individual queens within a time span of 16 h. Together, these results show that honey bee queens regularly produce eggs whose weight varies considerably. Here, we have shown that prenatal maternal investment in *Apis mellifera* is highly variable. I found that egg weight differed significantly between different queens ($F = 22.3$; $P < 0.001$) and among eggs laid by the same queen in different seasons. Older queens produced significantly lighter eggs than younger ones did ($F = 15.3$; $P < 0.001$). In spite of the differences between queens, variability of weight was also large within queens. In some cases, the weight of eggs laid simultaneously by the same queen varied by up to 70%. As shown in other species, the different maternal investment influences traits of offspring even in their adult stage.

I also observed that the effect of the nepotism on the context of queen rearing is expected because, among other things, polyandry in queens of the honey bee leads to many subfamilies within a colony (Getz and Smith, 1983). Worker bees play a nepotistic role during the queen-rearing phase by investing more resources into rearing those queen larvae which are genetically more closely related to them

(Page and Erickson, 1984; Koyama *et al*., 207). Several earlier studies have shown this, claiming that the individual fitness of the larvae offered to queenless colonies results in their being either reared as queens or being rejected (Hamilton, 1964; Wenseleers, 2007, Koyama *et al*., 2009).

Here, I show that relatedness plays practically no role but that individual attractiveness (as estimated by egg-weight measurements) is of great importance. Laidlaw and Eckert (1950) mention that the rearing of queens from old larvae might merely produce highly imperfect individuals with at least some worker characteristics. The race and strain of bees and the size of the colony greatly affect the number of queens reared by a colony. These authors further mention that the Italian and *Caucasian* races tend to produce fewer queen cells than other races such as the *Carniolan*. Król (1974) and Mohammedi and Le Conte (2000) have stated that the different origins of larvae grafted and nurse bees influence larval acceptance. In contrast, Breed *et al*. (1984), Guler and Alpay (2005) and Albarracín *et al*. (2006) found that the racial origin of larvae reveals no significant difference in the acceptance percentage of grafted larvae.

Concerning the acceptance percentage of related versus unrelated grafted larvae, this study has revealed no significant preference for related larvae by queen-rearing workers (n = 450, χ^2 = 0.76, P = 0.38). In other words, nurse bees do not functionally discriminate between related and unrelated larvae during queen rearing (Tarpy and Fletcher, 1998). Breed *et al*. (1984) have concluded that workers are incapable of discrimination among larvae on the basis of relatedness. Visscher (1986) has studied kinship discrimination by using larvae and eggs for queen rearing. In the first experiment, he transferred larvae into royal jelly in queen cups and observed a relatively high acceptance (57%) with no evidence of nepotism; the bees reared nearly equal numbers of unrelated larvae and related nestmate larvae (77 and

76 respectively). In a second experiment – in which eggs rather than larvae were transferred – the acceptance percentage was considerably lower (13%) with a significant preference for nestmate over foreign-hive eggs. On the other hand, Page and Erickson (1984) suggest that adult honey bee workers have the ability to discriminate among their own highly related nestmate siblings over less-related non-nestmates. Tarpy *et al*., (2004) have reported that, when colonies rear queens, a small amount of conflict might occur. This would involve the question whether or not those individuals to be raised as queens is based on genetic relatedness, but the workers still usually cooperate in constructing queen cells so that the queens which do emerge are of high reproductive quality.

Another result of this study is worth underlining here. From hatching conditions, we can exclude any influence of colony odour. I provide evidence that choosing the strongest larvae instead of those with the strongest relatedness to other group members might help to reduce intra-colonial competition. Consistent with DNA data analysis of nurse bees and larvae, our findings re-emphasize that no evidence exists for kin discrimination in intra-colony behaviour during queen rearing. However, some degree of overlap must be present in the attractiveness cues for royalty of larvae from certain patrilines. Our data also support the hypothesis that the worker bees do not take into account the degree of relatedness, but rather the fitness potential of the larvae. This view is supported by findings that worker larvae derived from eggs laid by parasitic *Apis mellifera capensis* receive preferred royal treatment by European honey bee workers (Beekman *et al*., 2000). Workers of *A. m. capensis* are known to have an exceptionally high reproductive potential (Jordan *et al*., 2008). However, in contrast to many studies involving other species (Torres-Vila and Rodrı´guez-Molina 2002), we have observed for example the following trend. The expected relationships (positive in the case of queen weight and number of ovarioles,

and negative in the case of the pre-oviposition period) between egg-weight and queen traits tend to affect their reproductive potential. However, none of the relationships was found to be significant. The lack of significance is probably caused by the non-representative samples that we have analysed. Because nurse bees prefer larvae from heavier eggs, our queen sample is somewhat lacking in regard to individuals with the detrimental effect of low maternal investment. This artefact tends to reduce the covariance between traits. This result does not disprove Hamilton's rule; it merely stresses that relatedness is just one factor in his equation (Wilson, 2005). Nowadays, we face strong challenges in discussing all of the theories addressed by Nowak *et al*. (2011). Many arguments (see Abbot *et al*., 2011; Boomsma *et al*., 2011; Strassmann *et al*., 2011; Ferriere and Michod, 2011; Herre and Wcislo, 2011) have been proposed in answer to this paper by Nowak *et al.* who have attempted to provide a model for the evolution of eusociality behaviour. On the other hand, their arguments are based upon a misunderstanding of evolutionary theory and a misrepresentation of the empirical literature. Moreover, the challenge of Nowak *et al*. has proposed connections between relatedness and the evolution of eusociality. For instance, they dismiss the utility of Hamilton's insight that relatedness has a profound evolutionary effect, formalized in his widely accepted inclusive fitness theory as Hamilton's rule.

Under natural conditions, all offspring are indeed related within a honey bee colony, but to very different degrees. This indicates that a superior halfsib (r = 0.25) probably represents the reproductive interest better than one which is three times more closely related but not as well as one which is a possible suitable super-sister (r = 0.75). However, whether or not this principle is driven by an avoidance of the negative influence on fitness through patriline competitions and/or by a dominant impact of superior maternal investment on the reproduction of the new queens

remains unknown. Consequently, these investigations have revealed and confirmed that relatedness plays no role during the queen rearing during which I have observed candidate larvae queens and marked worker bees by monitoring. The present results can be summarized by stating that worker honey bees can indeed discriminate between queen larvae of three patrilines within the same hive. But they do not use this information to prefer due to relatedness. If larvae from rare subfamilies are preferentially reared to become queens, then two possible explanations can be suggested. The first is that larvae which workers preferentially raise are most frequently those which belong to the subfamily with the highest frequency. Although we cannot exclude this mechanism, neither a theoretical reasoning nor any empirical evidence supports such a mechanism. There is always a numerical dominance due to unequal numbers of spermatozoa contributed by drone fathers. To avoid this problem, our experimental procedure was designed by utilizing exactly 1.0 µl from each drone. The second explanation is based on the variation in the reproductive traits among the fathering drones. The results here also reveal that the frequency of care behaviour towards each queen larva differs with regard to the patriline derived from three different drones. Meanwhile, as has been shown elsewhere, chemical signalling is the most important mode of communication between individuals in insects and this has been widely studied in several orders, mainly in the contexts of sexual selection, social recognition and regulation (Wyatt, 2003). However, the study of chemical communication in the context of interactions between brood and adults in social insects has remained fairly unexplored and might reveal important new mechanisms of adults care regulation. For instance, honey bee (*Apis mellifera*) larvae produce a brood pheromone which influences the care behaviours of workers, suggesting a possible role of the brood pheromone as a begging signal. The brood

pheromone not only influences worker behaviour, it also has lasting consequences for their physiology (Le Conte *et al*., 2001).

The lack of nepotism even at the individual basis may support the hypothesis of Nowak *et al.*, who found that relatedness is not important for the evolution of eusociality, and that it is more likely the result than the cause of cooperation. But there is evidence to assume that the use of eusocial societies does not represent a proper model to answer this question. At the beginning of the evolution of sociality, where individuals start to cooperate at different levels, kin recognition and nepotism were essential to increase their fitness and later, when the sisterly rearing appeared, their inclusive fitness (Gadagkar, 1990). However, since eusociality and consequently worker sterility have developed, available broods for queen rearing no longer provide the alternative 'related or unrelated', but rather 'more related or less related'. The ability of worker bees for nepotism at this stage of evolution is likely to reverse the former advantage into its opposite. Nepotism within a related colony favours intra-colonial competition, with negative impacts for all members of the unit. In addition, within a related group, solely the degree of relatedness is a very inappropriate criterion for selection, because it may lead to a wrong decision – even for most closely-related individuals – to select one. Our data provide evidence that worker honey bees draw their conclusion from this fact and thus transform their kin recognition ability into an aptness to assess the ability of young larvae to develop into successful gynes. The large genetic (multiple) mating and an environmental (prenatally very different) supply provide a good platform for this (AL-Kahtani *et al.,* submitted).

Another result of the current work is worth mentioning here. One can speculate that the preference of individuals for more closely related kin is still present but that it is attributable to the necessary cooperation of hundreds of other colony

members; in gyne rearing, this preference is masked and unlikely to translate into a colony decision. Up to now, only the final outcome of the cooperation has been viewed. Consequently, we have also observed the behaviour of nurse bees towards larvae in a queenless colony. Using DNA fingerprinting, we investigated the relatedness of larvae and nurse bees which are involved in the rearing process of these larvae. In other words, we examined the link between the exposure of Nasonov glands (ENGs) and the recruiting of special larvae to be reared as queens. Behavioural observations within a hive are likely to disturb honey bees. However, in comparison with the observation hives used to date, the infrared video technique here provides a sensitive method for the long-term recording of honey bee activities in the natural darkness found inside a hive.

To our knowledge, the current experiment is the first one to show that ENG is used within the hive in *Apis mellifera.* The design of the observational unit and the individual of worker bees have provided additional information on individual behavioural patterns. Sladen (1901) first suggested that the odour of the Nasonov gland is involved in the orientation of honey bees; this was subsequently confirmed for orientation at the nest entrance (Butler and Calam, 1969), in swarm clustering (Morse and Boch, 1971), and at water collection sites and flowers (Free and Williams, 1970).

For the following reasons, our findings under natural conditions support the hypothesis that the pheromone of the Nasonov gland has a specific indoor role in enabling individual worker bees to campaign for special larvae to be reared as gynes. 1) ENG has been found to be performed exclusively at brood cells containing larvae suitable for queen rearing. Moreover, ENG is able to attract other bees and successfully recruit specific larvae. 2) A highly significant concentration of ENG is observed in cells later developing into queen cells. These results support the

assumption that ENG is not a general trigger for brood care but that it is involved specifically in campaigning for larvae to be reared as gynes. It is likely that not every single act of recruiting behaviour results in a group decision pertaining to foraging, nest maintenance and reproduction. The lack of a significant difference between the duration (assumed to be an indicator of the intensity of recruiting of ENG) of cells accepted for queen rearing after ENG and the highly significant worker density after recruiting for those cells which are not accepted provides strong evidence that differences in the intensity of recruiting do not influence the final group decision, whereas differences in the attractiveness of the preferred larvae do seem to influence it. If the decision of the recruiting bee for an individual larva corresponds with the notion of the group, then the larva's superior attractiveness is likely to fine-tune the search for it within the pheromonal sphere of the recruiting worker. However, the larval characteristics which initiate the ENG of individual bees and which subsequently result in group decision-making for the selection of specific larvae as queens remain unknown.

To sum up, we believe that the results of this study may be useful in regard to certain applications, for instance in queens' rearing production. For commercial queen producers, a reduction of the breeding queens' egg-laying to a smaller area may result in a smaller number of eggs and thus in consequence heavier eggs and likely fitter queen offspring. In the meantime, in order to diverge from the most commonly used methods by which young brood is reciprocally transferred between colonies, I utilized an artificial rearing of larvae which is capable of avoiding not only relatedness, but also another factor which may impact the acceptance for queen rearing colony. This is odour; it is in part environmental, and it can be differentiated by worker bees. Furthermore, the procedure for rearing eggs into viable larvae inside

an incubator may also be useful for rearing cryopreserved or genetically modified embryos (Manuscript 4).

4 General conclusion

Polyandry is common among highly eusocial insects, because it increases phenotypic variation within the colonies, which was shown to increase fitness. However, genetic variation within colonies is assumed to provide conflict within colonies, because worker bees may act nepotistically during the queen-rearing phase by preferring more closely related larvae. The data presented in this study did not show that relatedness is important for the selection of larvae for queen rearing. However, different maternal investments into the egg can significantly affect the chance of the resulting larvae to receive royal treatment or not. Egg weight, measured as a criterion for maternal investment differed greatly even within clutches laid simultaneously. Worker bees recruit for specific larvae by a specific behaviour, the exposure of the Nasonov gland. However, this behaviour is not guided by relatedness, but in addition to egg size by genetic differences within a colony assumed to be linked with differences in larvae attractiveness. Under natural conditions, all members of a colony are more (r = 0.75) or less (r = 0.25) related. Consequently, genetically or environmentally-driven attractiveness – but not relatedness – represent the key criteria for selecting larvae for gyne production. Considering also the other factors (cost and benefits) in Hamilton's (1964) equation, the sole preference for a higher relatedness is not necessarily the best option for inclusive fitness. Consequently, in social societies, absence of the preference for a higher relatedness does not support the new hypotheses of Nowak *et al*. (2010) that relatedness is irrelevant for the evolution eusociality.

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Declaration

I hereby declare that the work contained in this thesis is my own and contains the results of an original investigation, except where otherwise referenced or acknowledged. This work was carried out while I was enrolled as a student for the degree of Doctor of Philosophy in the Institute for Bee Research, Hohen Neuendorf. Humboldt University of Berlin, Germany, This thesis has not been previously submitted for examination at this, or any other, university.

Saad Naser AL-Tameem AL-Kahtani

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