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Original article

A new method for rearing genetically manipulated honey bee workers[1]

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Abstract – Advanced functional genomic research on the honey bee (*Apis mellifera*) will require methods that allow researchers to work with bees derived from genetically manipulated embryos. In vitro rearing of honey bees is laborious, and it is often difficult to obtain individuals that span a normal phenotypic range. We present a technique that allows manipulated honey bee eggs to be introduced into hives so the larvae can be reared in a colony setting. Newly laid eggs on removable cell bases were injected with nuclease free H_2O, double-stranded RNA (dsRNA), or left untreated. They were inserted into specially designed hives where they hatched. Colonies accepted a satisfactory proportion of eggs from all treatment groups (28–53%). Further, a set of physiological and morphological traits (i.e., total protein in the hemolymph, head width, antennal length, and the length of a compound vein) were compared between workers derived from untreated, incubated eggs, and bees that naturally emerged in the hives. No significant differences were found between the groups. Our method therefore overcomes the challenges associated with in vitro rearing.

***Apis mellifera* / rearing protocol / laboratory hive / functional genomic research**

1. INTRODUCTION

The perspective and interest for functional genomic research on the honey bee (*Apis mellifera* L.) are expected to expand with the release of the honey bee genome sequence (see http://hgsc.bcm.tmc.edu) and the development of new molecular methods. The honey bee worker has been used to study the molecular basis of learning and memory (Grohmann et al., 2003; Scheiner et al., 2004), the regulation of social behavior (Page and Erber, 2002; Grozinger et al., 2003; Whitfield et al., 2003), and aging (Amdam et al., 2004a). Currently researchers are developing techniques for conducting reverse genetics on honey bees (Robinson et al., 2000; Beye et al., 2002; Amdam et al., 2003).

Researchers may be unable to exploit the potential of the honey bee as a genetic model organism without new tools that allow adult bees to be raised from genetically manipulated embryos (Beye et al., 2002; Amdam et al., 2003). Techniques such as microinjection and enucleation require that honey bee eggs of known age are staged in a laboratory setting (Omholt et al., 1995), but this is challenging because the queen will only lay eggs in the milieu of the hive. Further, the eggs adhere to the bottom of wax cells, which makes them difficult to remove. Eggs of known age may be obtained by caging a queen on a comb, but the disturbance caused by caging often interferes with the resumption of normal egg laying (Omholt et al., 1995).

Rearing honey bee larvae after they hatch is an obstacle to the use of honey bees for molecular genetics research. In the hive, the rearing of a worker honey bee requires hundreds of

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feeding visits by nurse bees. Honey bee workers can be reared to adulthood in vitro (Rembold and Lackner, 1981; Shuel and Dixon, 1986; Czoppelt and Rembold, 1988; Peng et al., 1992), but these techniques are laborious and the resulting bees may vary greatly in weight and morphology (Michael and Abramovitz, 1955; Rembold et al., 1974, 1980; Shuel and Dixon, 1986). In vitro rearing is therefore inappropriate in cases where it is necessary to detect subtle phenotypic differences between mutants and wild type bees.

We have developed a method that overcomes the challenges associated with in vitro rearing by making use of specially designed laboratory hives. The protocol is simple, allows for the production of large numbers of workers, and the adult bees that are produced appear to be physiology and morphology normal. The technique is likely to be useful in cases where the age of the embryo is critical and where the mutant phenotype has a low penetrance during the larval stage.

2. MATERIALS AND METHODS

2.1. Bees

Colonies were obtained from a mixed stock of honey bees (*A. m. mellifera* and *A. m. carnica* hybrids) kept in the apiary of the Norwegian University of Life Sciences, Aas.

2.2. Laboratory hives

Egg collection hives were constructed as described by Omholt et al. (1995). The design was modified so that a hive consisted of a single hive box with 2 aluminum frames of the same size as Norwegian standard frames (370 × 255 mm). Each of these frames held 6 Jenter frame modules (Karl Jenter, Nurtingen, Germany) that were loosely fitted into the frames (Fig. 1). This allowed individual frame modules to be removed and inserted. For each module, the wax-built side with brood cells was facing inwards, and 90 extractable Jenter cell bases were mounted into drilled holes and stabilized by a small aluminum grid (Fig. 1). This allowed us to collect and introduce eggs by extracting and inserting individual cell bases, as the bases were easily accessible from the side of the frames that made out the outer walls of the hive. For further information on the egg collection hive design, see Omholt et al. (1995).

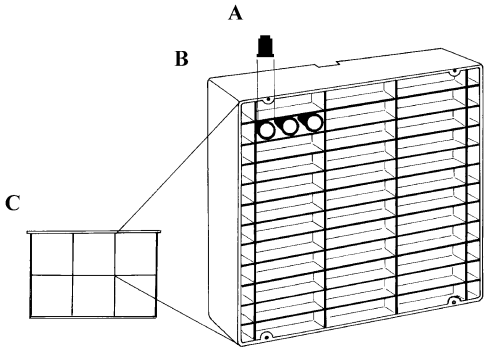

Figure 1. Graphical representation of the hive-units that were modified relative to the laboratory hive design of Omholt et al. (1995). A: one of the 90 extractable Jenter cell bases that were mounted into each frame module, B: the removable Jenter frame module, rear view, with an inserted aluminum grid with cells that each held 3 cell bases, and C: the surrounding aluminum framework (370 × 255 mm). Six frame modules could be mounted into this unit. One laboratory hive consisted of 2 fully mounted 370 × 255 mm frames, which were accommodated in a wooden hive body where they were separated by 12 mm bee space. The frames were inserted into the hive body so the Jenter modules' wax-built side with brood cells faced inwards. The cell bases (A) were then easily extractable from the outer walls of the hive.

The laboratory hives were kept in an indoor flight room (Nye, 1962; Jay, 1964; van Praagh, 1972). The bees were offered sugar syrup (40%) and pollen from freestanding feeders. In addition, the colonies were provided with pollen dough through the crown boards of the hives (as described by Omholt et al., 1995).

2.3. Experiment 1: Acceptance of eggs for rearing

Eggs (0–6 h old) were collected over a period of three weeks by extracting cell bases with adhering eggs. Each day, the newly collected eggs were divided into three groups. One group was left untreated. The other two received injections in situ (i.e., while on the Jenter cell base) with nuclease free H_2O and double-stranded RNA (dsRNA) for vitellogenin (GenBank: AJ517411), respectively. Injection of dsRNA causes RNA interference (RNAi, degradation of corresponding mRNA) in bees (Beye et al., 2002; Amdam et al., 2003). Vitellogenin RNAi was chosen as a treatment because it has little or no penetrance during the larval stage (Amdam et al., 2003). The dsRNA was produced as described by

Amdam et al. (2003), and all injections were performed as described by Beye et al. (2002), with an average amount per embryo of 300 picoliter.

The eggs were incubated at 35 °C and 80% RH while still adhering to the cell base. A solution of 16% H_2SO_4 was used as humidity stabilizer. After 65 h, the eggs were inserted into the laboratory hives in batches of 40–80 per hive. In each case, groups of 10–20 eggs were introduced in close proximity to each other. The cell bases that occupied these locations before the manipulated eggs were inserted had been cleaned the previous day. Therefore, the incubated eggs either replaced newly laid eggs (0–24 h old), empty cell bases, or cell bases with some pollen or honey. The in-hive coordinates of the introduced eggs and the original content of the cell bases they replaced were recorded. In total we introduced 400 H_2O injected eggs, 400 dsRNA injected eggs, and 1375 untreated eggs.

The cell bases were taken out and examined two days after the introduction. An egg was characterized as accepted if its cell base contained a larvae surrounded by brood food. Two sets of cell bases containing untreated introduced eggs ($n = 100$ for each set) were not examined, and remained undisturbed in their nursing colonies.

2.4. Experiment 2: Comparison of adult bee phenotypes

The Jenter frame modules that contained the sets of untreated eggs (i.e., non-injected introduced embryos) that remained in nursing colonies after the completion of Experiment 1 were removed from the hives after 18 days. Brood not located at the in-hive coordinates of the untreated eggs was removed from the modules. The modules were then incubated at 35 °C and 80% RH for 2 days, and the bees that emerged were collected. The remaining sealed brood was discarded. The rationale behind this procedure was as follows: for the cell bases where the untreated eggs were inserted but not accepted, the replacement brood had to be at least three days younger than the experimentally introduced brood, which was 65–71 h old at the time of introduction. Thus, by collecting bees during a restricted time interval (20–22 days after the experimental eggs were laid, equaling the developmental time of honey bee workers ± 1 day), we maximized the probability of obtaining bees that derived from the introduced eggs only. Controls were obtained from a separate set of Jenter frame modules where bees developed from eggs that were not manipulated in any way; i.e., they were never extracted, incubated or injected.

Bees from injected eggs were not used for this experiment because putative physiological and morphological differences between injected bees and control could derive from a mechanical disturbance of the embryo as well as a difference in rearing. Evaluation of such treatment effects were not within the scope of our study.

2.5. Physiological and morphological assays

The adult workers (experimental bees and controls) were either sampled at emergence or marked with a spot of paint on the thorax. These marked bees were introduced into a separate hive and sampled when 5 days old.

2.5.1. Total hemolymph protein

Total hemolymph protein was measured because it is a good indicator of the physiological state of a bee (reviewed by Amdam and Omholt, 2002), and because the hemolymph protein level of a newly emerged bee is affected by its rearing environment (reviewed by Amdam et al., 2004b). In older bees, the protein level is an indicator of the behavioral state of the workers (Engels and Fahrenhorst, 1974).

Bees were immobilized at 4–8 °C, and the hemolymph (2 μL) was extracted with Drummond micropipettes (Fisher Scientific) by puncturing the abdomen between the 3rd and the 4th tergite using a sterile 0.5 mm Neolus needle (BD). Care was taken to avoid contaminating the samples with tissue fragments and foregut content from the bee. The concentration of solubilized protein in 1 μL hemolymph was determined twice for each individual by the BioRad Protein Assay (BioRad) at 595 nm.

2.5.2. Morphology

Head width, antennal (scape) length, and the length of a compound vein (united radius and media vein, characters as described by Snodgrass (1956)) were used to compare the morphology of workers derived from introduced eggs and controls (Hartfelder and Engels, 1992; Radloff and Hepburn, 2000). The antenna and wings were mounted on glass slides, and the head was arranged on a piece of styrofoam. The measurements were performed using a stereo-microscope (Leica) with an ocular measurement scale.

2.6. Statistical analyses

The acceptance of the inserted eggs was calculated as a proportion for each clustered groups of 10–20 eggs. The acceptance for each treatment group was then estimated as the mean of these proportions. We tested the effect of treatment on acceptance by assigning a categorical level of manipulative action to each treatment group. The untreated, incubated

Table I. Physiological and morphological characteristics of honey bee workers stemming from untreated introduced eggs (Manipulated) and of bees that naturally emerged in the laboratory hives (Control). A: newly emerged bees, $n = 19$ and $n = 20$ for Manipulated and Control, respectively. B: 5-day-old bees, $n = 30$ except where noted.

Variables	Mean ± SE Manipulated	Mean ± SE Control	*P*-values*
A			
Protein titer (μg/μL)	6.20 ± 0.32	6.75 ± 0.47	0.34
Head width (mm)	5.81 ± 0.01	5.83 ± 0.01	0.17
Vein length (mm)	7.26 ± 0.02	7.29 ± 0.02	0.29
Scape length (mm)	4.98 ± 0.02	4.97 ± 0.01	0.62
B			
Protein titer (μg/μL)**	12.40 ± 1.44	10.25 ± 1.20	0.26
Head width (mm)	5.84 ± 0.01	5.83 ± 0.01	0.49
Vein length (mm)	7.68 ± 0.02	7.73 ± 0.02	0.30
Scape length (mm)	4.94 ± 0.03	4.96 ± 0.02	0.58

* By a two-sided Student's *t*-test, ** $n = 10$.

group was assigned the lowest level, whereas the dsRNA injected group was assigned the highest level. The data were analyzed as a one-way ANOVA, and putative differences between treatments were determined using a Fisher post-hoc test. Further, the proportion of accepted eggs per clustered group was regressed on the proportion of cell bases at the same in-hive coordinates that originally held eggs (see Sect. 2.3). Residuals were plotted against the corresponding fitted values to detect putative model inadequacies. The putative differences between the physiological and morphological characteristics of bees stemming from inserted eggs and control workers (Exp. 2) were tested using a multivariate *t*-test (Hotelling's T^2), the multivariate extension of the Student's *t*-test. The null hypothesis was that the group means for all response variables were equal. The analyses were performed with Statistica 6.0.

3. RESULTS

3.1. Experiment 1: Acceptance of eggs for rearing

There was a significant effect of treatment on the acceptance of the introduced eggs (ANOVA, $F_{2,87} = 5.9$, $P < 0.05$). The untreated and H_20 injected eggs were accepted at equal proportions as 0.53 ± 0.04 (mean ± SE, $n = 50$) and 0.44 ± 0.06 ($n = 20$), respectively (Fisher post-hoc test: $P = 0.17$, df = 87). The acceptance of the dsRNA injected eggs however, were significantly lower (0.28 ± 0.05, $n = 20$, Fisher post-hoc test: $P < 0.05$, df = 87). Further, the proportion of cell bases that contained eggs before the introduction of the manipulated embryos had a significant (ANOVA, $F_{1,88} = 36.2$, $P < 0.005$) positive effect ($r = 0.72$, $n = 90$) on acceptance. The residual plots did not reveal any systematic patterns that could flaw the analysis (See Montgomery and Peck, 1992 for further details).

3.2. Experiment 2: Comparison of adult bee phenotypes

We found no significant differences for any of the tested characters when we compared workers that derived from the introduced untreated eggs, and workers that developed from eggs that were never manipulated ($T^2 = 4.7$, $P = 0.47$ and $T^2 = 4.9$, $P = 0.54$ for the 0-day-old and 5-day-old workers, respectively). Descriptive statistics are given in Table I. Note that the mean protein titer of the 5-day-old workers is higher than that of the newly emerged bees. This is normally observed in honey bee workers (Engels and Fahrenhorst, 1974; Amdam et al., 2004b).

4. DISCUSSION

As molecular biologists working with traditional model organisms such as *Drosophila*

melanogaster make advances, it is necessary for researchers working with non-traditional model organisms to apply these advances to their own organism by creating new methods and procedures. We have developed a modified hive that allows for the manipulation of honey bee embryos. We have demonstrated that manipulated honey bee eggs are accepted by nursing colonies and that they appear to develop into normal bees. Introductions of vectors and other genetic materials into honey bee embryos, in most cases, require considerable manipulations of young eggs (Beye et al., 2002; Amdam et al., 2003). Our laboratory hive facilitates collection and introduction of eggs. Moreover, entire sections of sealed brood can be recovered by removing modules from the outside walls of the hives. We think this new design represents a substantial contribution in the endeavor to make the honey bee a laboratory animal suitable for functional genomic research.

The H_20 injected and untreated incubated eggs were accepted in equal proportions in our study. The dsRNA-injected eggs, however, seemed to be rejected at a higher rate. Data from Amdam et al. (2003) indicate that early mortality of dsRNA injected embryos is higher than for embryos injected with H_2O only. This may, at least partly, explain why we recovered significantly fewer larvae in the dsRNA treatment group. Further, the negative effect of vitellogenin RNAi on survival might indicate that the vitellogenin gene product has a possible function during early development.

The success of our method will depend upon the specific manipulation to the embryos. The use of expression vectors or knock-downs constructs that are designed to target the adult phenotype may influence the larval phenotype as well, and might result in the death of the larvae or in the production of sub-viable adults. It is encouraging to note though, that eggs injected with vitellogenin dsRNA are well accepted, and that they appear to develop into adults that are normal except for their mutant phenotype (Amdam et al., 2003, this study was performed by our group using a preliminary version of the protocol presented here).

We found that the proportion of cell bases that contained eggs before the manipulated embryos were introduced had a significant effect on acceptance. This implies that an egg that replaced another egg was more likely to be accepted than an egg that replaced an empty cell base or a cell base that contained a droplet of honey or a little pollen. Our observation is in line with results showing that grafting of larvae into queen cells previously occupied by brood ("double grafting") improve acceptance (Weiss, 1983). However, eggs, not larvae, were exchanged in our experiment, and our finding may be a simple consequence of workers selectively cannibalizing eggs in cells regarded as unfit for the rearing of brood. An alternative explanation is that queen-produced egg pheromones that suppress policing (Ratnieks, 1992, 1995) are deposited on the cell walls by laying queens. In this connection, it is interesting to note that honey bee colonies vary in their acceptance of worker laid eggs (Oldroyd et al., 1994) and mite-infested brood (Spivak and Reuter, 2001). It is therefore possible that colonies also differ in their readiness to accept manipulated eggs. We could not design an experiment that would address this question sufficiently because our indoor flight room only accommodated a small number of hives. However, it is not unreasonable to believe that the genotype of both the egg and the nursing colony may affect the acceptance of a manipulated embryo, and future studies might determine whether traits that facilitate acceptance can be selected for.

All in all, our results indicate that the protocol presented here is useful for production of mutant workers when altered phenotypes have a low penetrance during the larval stage. In cases where the genetic manipulation results in non-viable pupae or adults, the method is probably of minor utility. The technique seems to overcome the main challenges associated with in vitro rearing, and this is encouraging because the need for practical tools for rearing genetically manipulated honey bees is likely to increase over the next few years.

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Résumé – **Nouvelle méthode pour élever des ouvrières d'abeilles (*Apis mellifera*) manipulées génétiquement.** Le développement et l'usage largement répandu de l'abeille domestique (*Apis mellifera* L.) comme organisme modèle d'étude nécessitent des méthodes qui permettent aux chercheurs de travailler avec des ouvrières adultes issues d'embryons génétiquement manipulés. Les protocoles d'élevage in vitro sont prévus pour des ouvrières d'abeilles et des reines mais peuvent être imparfaits si une faible variance interindividuelle ou un grand nombre d'abeilles sont nécessaires. Nous présentons ici un nouveau protocole pour l'élevage des ouvrières. L'utilisation d'un modèle de ruche mis au point pour le travail au laboratoire nous a permis de récolter, de manipuler et d'introduire des centaines d'œufs sans ouvrir les ruches durant ces procédés. Les œufs fraîchement pondus ont été récoltés et ont reçu une injection d'eau sans nucléase (lot 1), ou d'ARN à double chaîne (ARNds) (lot 2) ou sont restés non traités (lot 3). Ils ont été maintenus en étuve durant 65 h, puis introduits dans les ruches de laboratoire où ils ont éclos. Nous avons noté 2 j plus tard si l'œuf avait ou non été accepté. En outre, les caractéristiques physiologiques et morphologiques d'abeilles âgées de 0 à 5 j issues d'œufs maintenus en étuve et non traités ont été comparées à celles d'ouvrières qui étaient nées naturellement dans les colonies (témoins).
Les colonies ont accepté un pourcentage satisfaisant d'œufs des trois lots (0,44 ± 0,06 ; 0,28 ± 0,05, et 0,53 ± 0,04, respectivement), mais l'acceptation des œufs du lot 2 (injection d'ARNds) a été significativement plus faible que celle des deux autres lots (test de Fisher post-hoc : $P < 0,05$, df = 87). Les caractéristiques physiologiques et morphologiques des ouvrières d'œufs manipulés, à savoir la teneur totale en protéines de l'hémolymphe, la largeur de la tête, la longueur de l'antenne (scape) et la longueur des veines radiale et médiane, n'étaient pas différentes de celles des témoins (Hotelling's T^2= 4,7, $P = 0,47$ et $T^2 = 4,9$, $P = 0,54$ pour les ouvrières âgées de 0 et de 5 j, respectivement). Les résultats montrent que notre méthode sera utile pour produire des ouvrières mutantes lorsque les modifications ne s'expriment pas encore dans le phénotype au cours de la période larvaire. La technique semble surmonter les principaux problèmes associés à l'élevage in vitro et c'est encourageant, car le besoin d'outils pratiques pour élever des ouvrières génétiquement manipulées augmentera vraisemblablement dans les prochaines années.

***Apis mellifera* / élevage in vitro / méthode / ruche de laboratoire / génomique fonctionnelle**

Zusammenfassung – **Eine neue Methode zur Aufzucht von genetisch veränderten Arbeiterinnen der Honigbienen.** Die Entwicklung und Nutzung der Honigbiene (*Apis mellifera* L.) als Modellorganismus macht Methoden erforderlich, die eine Forschung mit adulten Tieren von genetisch veränderten Embryos zu ermöglichen. Eine Anleitung zur Aufzucht von Arbeiterinnen und Königinnen in vitro wurde bereits erstellt, aber diese Anleitung könnte sich als nicht ideal erweisen, wenn eine niedrige inter-individuelle Varianz oder eine große Zahl von Bienen benötigt wird. Deshalb erarbeiten wir eine neue Anleitung zur Aufzucht von Arbeiterinnen der Honigbienen. Eine zum Gebrauch im Labor entwickelte Beute ermöglichte uns, mehrere hundert Eier zu sammeln, zu manipulieren und wieder in Zellen zu geben, ohne das Volk während der Prozedur zu stören. Frisch gelegte Eier wurden gesammelt, bei je einer Gruppe wurde Nuclease freies Wasser oder ds RNA injiziert, eine Gruppe blieb unbehandelt. Sie wurden nach Haltung von 65 Stunden im Brutschrank in die Laborbeute eingesetzt, wo sie schlüpften. Wir kontrollierten 2 Tage später, ob die Eier angenommen worden waren. Außerdem wurden physiologische und morphologische Eigenschaften der 0 und 5-Tage alten Arbeiterinnen, die von unbehandelten, inkubierten Eiern stammten, mit Arbeiterinnen verglichen, die auf natürlichem Weg in den Völkern geschlüpft waren (Kontrollen). Die Völker akzeptierten eine zufrieden stellende Anzahl der mit Wasser bzw. mit dsRNA injizierten und der unbehandelten Eier (0,44 ± 0,06, 0,28 ± 0,05, und 0,53 ± 0,04,), aber die Akzeptanz der mit dsRNA injizierten Eier war signifikant geringer als bei den beiden anderen Injektionen (Fisher post-hoc test: $P < 0,05$, df = 87). Die physiologischen und morphologischen Eigenschaften, wie Gesamtprotein der Hämolymphe, Kopfbreite, Antennenlänge (Scapus) und die Länge der radialen und der medianen Ader, unterschied sich nicht zwischen den Arbeiterinnen von manipulierten Eiern und den Kontrollen (Hotelling's T^2 = 4,7, P = 0,47 und T^2 = 4,9, $P = 0,54$) für die 0 – Tage und 5 – Tage alten Arbeiterinnen. Diese Ergebnisse zeigen, dass unsere Methode zur Erzeugung von Mutanten aus Arbeiterinnen geeignet ist, wenn sich im Larvalstadium Änderungen im Phänotyp noch nicht ausprägen. Die Technik scheint das Hauptproblem zu überwinden, dass bei einer in vitro Aufzucht auftritt. Der Erfolg dieser Methode ist ermutigend, denn der Bedarf für die Aufzucht von genetisch manipulierten Honigbienen wird wahrscheinlich in den nächsten Jahren ansteigen.

***Apis mellifera* / Aufzuchtsanleitung / Laborbeute / funktionelle Genforschung**

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