Apidologie 29 (1998) 547–554

Original article

Biopsied preblastoderm honeybee embryos develop into normal honeybee queens

Ronglin Yu, Arne Hagen, Stig W. Omholt*

Department of Animal Science, Agricultural University of Norway,
P.O. Box 5025, 1432 Ås, Norway

(Received 22 January 1998; accepted 9 June 1998)

Abstract – Preblastoderm honeybee (*Apis mellifera*) embryos (8.5–9.0 h) were biopsied by extracting a small amount of ooplasm from the anterior part. Nearly 60 % of the embryos hatched into larvae and 45 % of these emerged as queens. It is shown that extraction of up to 80 nuclei is not likely to cause any morphological or behavioural abnormality in the adult queen. Beyond this number the survival rate declines rapidly. Combined with a technique for cryopreservation of ooplasmic fractions recently developed by us, we are now able to perform comprehensive testing or screening of adult honeybee queens, while having their totipotent embryonic nuclei stored in liquid nitrogen.

biopsy / honeybee embryo / nuclear transplantation / embryo cloning / cryopreservation

1. INTRODUCTION

The honeybee egg is about 1.6 to 1.8 mm long, with a maximum diameter of 0.35 mm, which gives an egg volume of about 135 nanolitre (nL) (approximately ten times that of a *Drosophila* egg [1]). The outer and inner egg layers (chorion and vitelline membrane) are about 0.1 and 0.25 μm thick, respectively [2]. The early development of the embryo involves ten synchronous cleavage mitoses where the resulting nuclei migrate into the peripheral egg layer during a parasynchronous 11th mitosis (8 to 9 h). Thereafter, a peripheral cell layer, the blastoderm, is formed as is the case for nearly all pterygote insects [3]. The complete embryogenesis is amenable to direct observation, as honeybee embryos become highly transparent when placed in paraffin

* Correspondence and reprints
E-mail : stig.omholt@ihf.nlh.no

oil, resolving individual cells and even nuclei [2]. The developmental period is normally 72 h at 35 °C.

An efficient nuclear transplantation methodology for honeybee embryos was recently described by us [7]. It was shown that nuclei from 8 to 9 h preblastoderm embryos are totipotent. Despite the lack of an efficient enucleation technique for honeybee embryos, we are now able to produce embryo cloned larvae and queens (i.e. larvae and queens expressing the nuclear donor genotype only) by this transplantation protocol. However, in addition to a nuclear transplantation protocol, an embryo cloning technology for honeybees (i.e. the total set of protocols or methods necessary for producing embryo cloned honeybee queens on a large scale), as an alternative or supplement to classical breeding work, will require protocols for efficient enucleation of young eggs of known age (< 2 h), and cryopreservation of totipotent nuclei extracted from young eggs (< 9 h). We have recently developed a method to cryopreserve totipotent preblastoderm nuclei by rapid freezing without the addition of a specific cryoprotectant or other additive [11].

Though not indispensable for a practical implementation of an embryo cloning technology, access to a biopsy (or nuclear aspiration) protocol allowing development of the manipulated eggs into normal queens (which are tested for actual traits), would be very convenient. Lack of such a protocol would imply killing of the young embryo and cryopreservation of all extracted nuclei from which cloned queens could be made later on, or the immediate use of a fraction of the extracted nuclei to make cloned individuals. Of these two strategies the former is the most efficient, but it will involve the making of cloned individuals for every mating one wants to test. This is not necessary if the biopsied eggs develop into normal queens that can be tested immediately.

In connection with a future genetic transformation technology of honeybees, biopsies from genetically transformed embryos would allow screening of these embryos before hatching. In addition, cryopreserved biopsies could be used to make individuals with the same genetic transformation and identical genetic background.

Here we report that embryos from which ooplasm is extracted at the preblastoderm stage develop into normal queens with no apparent morphological or behavioural abnormalities. Thus we are now able to perform comprehensive testing or screening of adult honeybee queens while having their totipotent embryonic nuclei stored in liquid nitrogen. Such a methodology has not previously been developed for any insect.

2. MATERIALS AND METHODS

Newly laid eggs, 3–15 min old, were collected from five unrelated artificially inseminated *Apis mellifera carnica* queens kept in a flight room. The eggs were collected from laboratory hives containing two sampling frames, each made from six Jenter frame bases (Karl Jenter, Nurtingen, Germany). Each Jenter frame contained 90 extractable cell bases made of plastic and coated with wax. Eggs deposited on these extractable cell bases were then collected without any disruption of the egg membranes or disturbance of the queen and the bees (for further details concerning handling of bees under flight room conditions and collection of eggs see [6, 7]). The eggs were incubated at 35 °C at 80 to 90 % relative humidity (RH) for 8.5–9.0 h (at this age the number of totipotent preblastoderm nuclei is at its maximum). A small amount of ooplasm was then extracted from each embryo at the anterior part with a transplantation pipette made from a borosilicate capillary tube (o.d. = 1.0 mm, i.d. = 0.58 mm) (Sutter Instrument Company, Novato, CA, USA), where the tip was bevelled to an angle of 15 degrees with a K.T. Brown Type Micropipette Beveller (Sutter Instrument Company, Novato, CA, USA), and then forged with a laboratory-made microforge to make the tip extra fine. The inner diameter of the pipette tip was 16 to 18 μm. The amount of ooplasm aspirated from each embryo varied in the range 4.6 to 12.0 nL. The measure was based on previous calculations of the volume of the pipette as a function of the distance from the tip.

All micromanipulations were performed with an Oxford micromanipulator (Singer Instrument Co., Roadwater, Somerset, UK), a microinjector (PLI-100, Medical Systems Corp., Greenvale, NY, USA), and a stereo microscope. The biopsy was performed by mounting ten plastic cell cups with one recipient embryo each [7] on modelling clay on the back side of a 90 mm Petri dish (counted as one series), and keeping the Petri dish at 30 to 35 °C and RH > 80 %, until the aspirations started. The aspiration of ooplasm was carried out at room temperature (20 to 30 °C), and we kept the RH above 70 % by letting the Petri dish with the eggs stay inside a moist plastic chamber accessible by one hand so that the dish could be properly orientated before each aspiration.

The aspirated ooplasm was immediately placed under a small drop of paraffin oil (Fluka 76 235).With an injection pipette (i.d. 2 to 3 µm) a small drop with 0.5 $mg \cdot mL^{-1}$ of the nuclear stain Hoechst 33 342 (Sigma) and 0.9 % NaCl, was injected into the ooplasm. After incubation at 35 °C for 30 min, the number of nuclei were counted using a Leitz Aristoplan microscope (Leitz Wetzlar, Germany).

The biopsied embryos were incubated for 63–64 h at 35 °C above a layer of 21 % H_2SO_4 (d = 1.15 or diluted 1:6.5) in closed plastic chambers, until hatching. To facilitate grafting, the larvae were placed in dram vial caps (small plastic caps) filled with 0.4 mL semi-artificial food mixture [9], and incubated for 24 h at 35 °C above a layer of 6.8 % H_2SO_4 (d = 1.05 or diluted 1:24) in a closed plastic chamber. Eggs serving as controls were collected from the same hive and incubated under the same conditions as the experimental ones.

Experimental and control larvae were then randomly grafted into artificial queen cups (Jenter system, Karl Jenter, Germany) and placed into a previously prepared queen-rearing colony. After capping, the queen cells were transferred to an incubator (35 °C and RH between 50 and 60 %) until emergence. All queens were weighed within 1 h after emergence.

Forty-three sister queens (20 controls and 23 experimental ones) were selected for further study. Nine control and 13 experimental queens were fixed in 70 % ethanol for morphometrical comparisons to detect developmental abnormalities in the adults caused by the micromanipulation of the embryos. Length and width of the fore wing and length and width of basitarsus III were measured according to [10]. The diameter of the spermatheca was measured after removal of its tracheoli, and the shapes of the sting and the mandibles were registered. Other characters could have been chosen to detect morphological abnormalities in the experimental group, but the ones chosen were considered to be sufficiently diverse and appropriate. The remaining queens were marked with a plastic tag on the thorax, and introduced together with about 1 000 young worker bees to Apidea mating hives (Transidea AG, Switzerland) for behavioural studies.

3. RESULTS

The number of nuclei in each extract from individual embryos varied from 0 to 106. The frequency distribution of extracted eggs as a function of number of nuclei extracted shows that less than 30 nuclei were extracted from most of the eggs (*figure 1*). Survival rate of the biopsied embryos during embryonic development was 59 % (95 % for controls), 80 % of the hatched larvae survived in vitro feeding (86 % for controls), and 55 % of the transferred larvae developed into queens (62 % for controls) (*table I*). This indicates that the majority of deaths caused by injuries from the manipulation are realised during the embryonic period.

There seems to be a significant inverse relationship between the number of nuclei extracted and the developmental potential of manipulated embryos (*figure 2*). But there is still a considerable survival rate in the 80–90 group.

The morphometrical comparisons between sister queens emerging from biopsied and non-biopsied embryos showed no significant differences (one way ANOVA, $P > 0.05$) between the mean values of the characters measured (*table II*). The sting was typically queen-like in all individuals, and all the mandibles were fully queen-shaped [8]. Of the ten experimental queens introduced into mating hives, seven succeeded in natural mating and started to lay eggs. Six of the ten controls did the same. All queens produced hundreds of workers while being kept in the Apidea hives in early

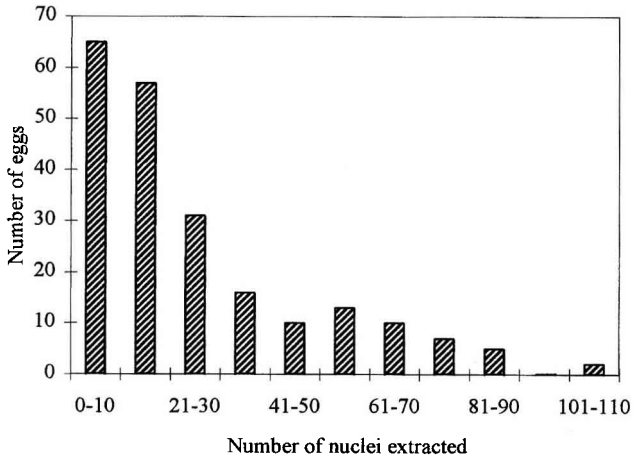

Figure 1. The frequency distribution of biopsied eggs as a function of number of nuclei extracted. The extraction volume was kept as constant as possible by the available equipment.

Table 1. Survival rates of the biopsied and non-biopsied embryos at different stages. The percentages refer to the values of the previous column. The surviving larvae were recorded after 24 h in vitro feeding.

Type	Initial	Hatching	Surviving	Adults
Controls	88	84 (95 %)	73 (86 %)	45 (62 %)
Biopsied	216	128 (59 %)	103 (80 %)	57 (55 %)

autumn 1996. Five experimental queens and three controls were randomly selected and successfully introduced into normal hives where they started production and later on were prepared for overwintering in September 1996. The experiment was terminated in June 1997. Due to the small number of colonies we found it futile to make high resolution measurements of colony characteristics. However, concerning honey yield and brood area it was impossible to distinguish between the two groups by visual means.

4. DISCUSSION

Apparently the mechanical injuries caused by the ooplasm extractions themselves, or the loss of ooplasm, seem to explain most of the reduced survival rate as only six embryos hatched out of 18 whose biopsies contained no nuclei. The results depicted in *figure 2* thus imply a proportionality between number of nuclei and amount of ooplasm extracted, which is quite reasonable. Furthermore, the result might indicate that the embryo more easily compensates for the loss of nuclei than the structural changes imposed by the micromanipulation and extraction of cytoplasm. On the other hand, it can not be excluded that some of the zero-nucleus biopsies are caused by sterile eggs that would not have developed in any case.

The hatching rate of biopsied embryos may be increased by extracting the nuclei

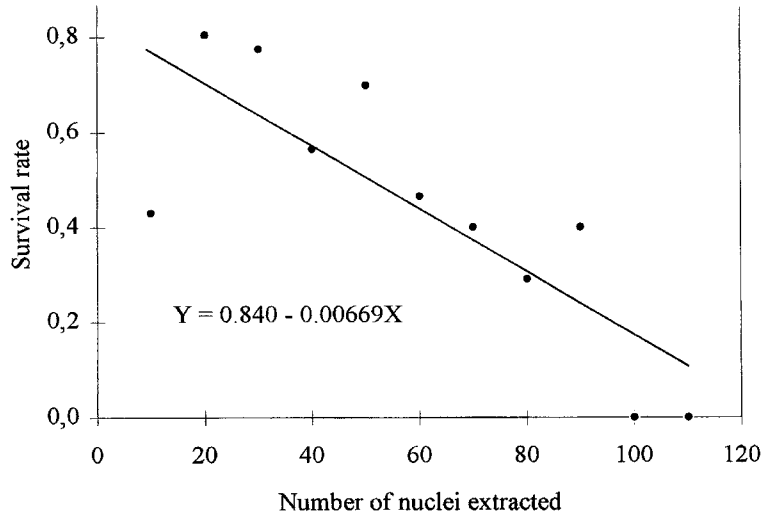

Figure 2. A linear regression analysis of the relationship between survival rate of eggs (Y) and number of nuclei extracted (X). The nuclear extraction records were sorted into 11 groups of ten. The inverse relationship is highly significant ($P < 0.005$).

Table 2. Morphometric comparison (mean ± sd) of unmated sister queens emerging from biopsied and unmanipulated eggs. The two groups were compared with a one-way ANOVA , and the mean values of the two groups were not found to be significantly different for any of the characters ($P > 0.05$).

	Character			
	Weight (mg)	Wing index (length/width)	Basitarsus III (length/width)	Spermatheca Ø mm
Experimental	220.13 (± 21.97)	3.069 (± 0.060)	2.129 (± 0.088)	1.076 (± 0.048)
n	21	13	13	10
Control	220.50 (± 25.31)	3.107 (± 0.069)	2.160 (± 0.090)	1.075 (± 0.049)
n	20	9	9	8

from a possibly less sensitive region than the anterior. However, as we are working with eggs deposited in their natural position attached to the Jenter base with their posterior end, we have found it very demanding to penetrate the eggs elsewhere without causing severe leakage. The nuclei themselves are about 5–10 μm, but they are surrounded by a cytoplasmic island of about 20 μm [2]. We have not tested the importance of keeping these islands intact, but if this is not crucial, the diameter of the pipette may be reduced to decrease mechanical injuries and thereby increase the hatching rate. The actual form of the pipette, however, seems to be quite good [7].

Based on our results so far, and presuming that an efficient enucleation methodology for eggs can be developed, we are of the opinion that an embryo cloning technology for honeybees is certainly within reach. Such a technology has several potential applications. From the scientific point

of view it can be used to study the relative importance of maternal cytoplasmic factors and mitochondrial DNA, compared to nuclear DNA, on larval and adult morphological and behavioural characteristics. If a genetic transformation technology for honeybees becomes a reality, the availability of cloned individuals with a fixed genetic background may simplify the unravelling of genetic regulatory structures considerably. Furthermore, several difficult nature/nurture problems related to social insect colonies in general might be attacked by use of cloned individuals.

It is premature to make an extensive elaboration of the commercial potential of an embryo cloning technology with regard to improvement of important production traits. This is an empirical issue that cannot be properly resolved before one has access to the technology. However, an outline of the possible benefits may be given. Due to lack of control of, and the large variation of, the production environment, honeybee breeding is a quite demanding enterprise compared to the case for other domesticated species. In fact, a comparison between the genetic potential for improvement of several production traits to what has actually been achieved by long term breeding programmes seems to confirm this rather strongly. One will be able to test groups of genetically identical queens mated within the same mating yard, or artificially inseminated with pooled semen, in several different environments at the same time. This might provide genetic information of considerable heuristic value for future selection work.

However, the most radical use of the technology will be to clone production queens that have shown superior performance for specific production characteristics. New genetic variation will be realised, tested and selected by use of breeding populations having a structure that for example might maximise heterosis. Such a scenario opens up the possibility for customisation of genotypes for specific environments. In this way honeybee breeding could become very similar to, for example, potato breeding.

One might object that the performance of honeybee colonies does not depend on the queen alone, but also on the worker patrilines and the interaction between these lines as well as with the queen. Thus, producing a cloned honeybee queen from a queen heading a genotypically superior colony will not give you a colony at the same level unless its spermatheca is filled with sperm identical to the sperm in that of the cloned queen. However, it can be validated theoretically that the overall genetic variance of a colony trait is likely to be dominated by the queen's genotype, firstly through her direct influence on the trait, secondly through the genetic covariance of the queen trait and the worker trait, and thirdly by contributing much more of the direct genetic variance of the workers compared to the drones [5]. Thus, we should be able to make a substantial improvement when making clones of honeybee queens heading superior colonies, even if we do not mate these clones with the same drones that mated with the selected queen genotype. On the other hand, if this conjecture turns out to be wrong, it seems possible in principle to circumvent the problem by making drones from sperm in a spermatheca [4]. Whether this will ever become a commercial reality remains to be seen.

ACKNOWLEDGEMENTS

We are grateful to two anonymous referees for pointing out some shortcomings of the original manuscript.

Résumé – Des embryons d'abeille biopsiés au stade préblastoderme donnent des reines normales. Bien que ce ne soit pas indispensable pour mettre en œuvre la technologie de clonage des embryons chez l'abeille (*Apis mellifera* L.), l'accès au pro-

tocole de biopsie, permettant le développement des œufs manipulés en reines normales (dont on teste les caractères réels), serait très pratique. Notre étude montre que des embryons dont l'ovoplasme a été extrait au stade préblastoderme donnent des reines normales sans anomalies morphologiques ni comportementales manifestes. Une petite quantité d'ovoplasme a été extraite de la partie antérieure d'embryons âgés de 8,5 à 9 h à l'aide d'une pipette de transplantation. La quantité d'ovoplasme aspiré a varié entre 4,6 et 12,0 nL par embryon. L'ovoplasme aspiré a été immédiatement recouvert d'une goutte de paraffine. À l'aide d'une pipette d'injection (diamètre interne 2 à 3 µm) une petite goutte d'une solution de NaCl à 0,9 % contenant 0,5 mg·mL^{-1} de colorant nucléaire Hoechst 33 342 a été injectée dans l'ovoplasme. Après incubation à 35 °C pendant 30 min, on a compté le nombre de noyaux sous un microscope Leitz Aristoplan. Les embryons biopsiés ont été mis à incuber pendant 63–64 h à 35 °C jusqu'à l'éclosion. Pour faciliter le greffage, les larves ont été nourries avec un mélange semi-artificiel et mises à incuber pendant 24 h à 35 °C. Les œufs servant de témoins ont été prélevés dans la même ruche et mis à incuber dans les mêmes conditions. Les larves témoins et les larves expérimentales ont été ensuite greffées au hasard dans des cupules artificielles (système Jenter) et placées dans une colonie possédant une reine et préparée à l'avance. Après l'operculation les cellules royales ont été transferrées dans une étuve (35 °C et 50–60 % HR) jusqu'à l'émergence. Le nombre de noyaux dans chaque extrait d'embryon a varié entre 0 et 106. Le taux de survie des embryons biopsiés a été de 59 % pendant le développement embryonnaire (95 % pour les témoins); celui des larves écloses et nourries in vitro de 80 % (86 % pour les témoins) et 55 % des larves transferrées ont donné des reines (62 % pour les témoins) (*tableau I*). Les comparaisons morphologiques entre reines sœurs n'ont pas montré de différences significatives pour les valeurs moyennes des caractères mesurées, qu'elles proviennent d'embryons biopsiés ou non (*tableau II*). L'aiguillon était typiquement celui d'une reine chez tous les individus et toutes les mandibules avaient la forme de celles des reines. Sur les dix reines expérimentales introduites dans des ruches de fécondation, sept, contre six des reines témoins, ont réussi à s'accoupler naturellement et ont pondu. Toutes les reines placées dans des ruches ont produit des centaines d'ouvrières.

embryon / biopsie / transplantation nucléaire / clonage / cryopréservation

Zusammenfassung – Nach Entfernung einer kleinen Menge Ooplasma aus dem Ei der Honigbienen entwickeln sich die Embryonen zu normalen Königinnen. Obwohl dies für die Entwicklung einer Technik zur Klonierung von Embryonen der Honigbienen nicht unbedingt erforderlich ist, wäre es ein Vorteil, Königinnen auf ihre Eigenschaften untersuchen zu können, aus deren Embryos zuvor Material zur Klonierung entnommen wurde. Hier berichten wir, daß sich Embryonen in normale Königinnen ohne deutliche Anomalien im Körperbau oder Verhalten entwickeln, aus denen Ooplasma im Stadium des Praeblastoderms extrahiert wurde. Mit einer Transplantationspipette wurde eine kleine Menge Ooplasma aus dem vorderenTeil von 8,5–9,0 Stunden alten Embryonen entnommen. Die Menge des extrahierten Materials schwankte zwischen 4,6 und 12,0 Nanoliter (nL) pro Embryo. Das abgesaugte Ooplasma wurde sofort unter einen kleinen Tropfen Paraffinöl gebracht (Fluka 76 235) und mit einer Injektionspipette (i.D. 2 bis 3 µm) ein kleiner Tropfen von Kernfärbung (5mg·mL^{-1} Höchst 3342 , Sigma, gelöst in 0,9 % NaCl) zugefügt. Nach einer Inkubation über 30 Minuten bei 35 °C wurde die Anzahl der Kerne unter einem Leitz Aristoplan Mikroskop (Leitz Wetzlar, Deutsch-

land) bestimmt. Die Embryonen wurden nach der Ooplasmaentnahme bis zu ihrem Schlupf 63 bis 64 Stunden bei 35 °C gehalten. Zur Erleichterung des Umlarvens wurden die Larven mit einer halb natürlichen, halb künstlichen Futtermischung versehen und für weitere 24 Stunden bei 35 °C inkubiert. Für die Kontrolle wurden Eier aus demselben Volk genommen und unter den gleichen Bedingungen gehalten wie die Versuchsgruppe. Die Versuchs- und Kontrolllarven wurden in zufälliger Reihenfolge in künstliche Weiselzellen umgelarvt (Jenter System, Karl Jenter, Deutschland) und in ein vorbereitetes Zuchtvolk gegeben. Nach dem Verdeckeln wurde die Weiselzellen bis zum Schlupf in einem Brutschrank (35 °C und rel. Luftfeuchte zwischen 50 und 60 %) gehalten. Die Zahl der Kerne aus dem Extrakt der einzelnen Embryonen schwankte zwischen 0 und 106. Die Überlebensrate der manipulierten Embryos während der Embryoentwicklung betrug 59 %, die der Kontrollen 95 %. Von den Versuchslarven überlebten 80 % nach dem Schlupf (86 % der Kontrollen). Von den umgelarvten Versuchslarven entwickelten sich 55 % zu Königinnen (62 % bei den Kontrollen). Der morphometrische Vergleich zwischen den Geschwisterköniginnen, die aus manipulierten und nicht-manipulierten Eiern schlüpften, zeigten keine signifikanten Unterschiede in den Mittelwerten der gemessenen Eigenschaften (*Tabelle II*). Der Stachel und die Mandibeln hatte bei allen Tieren die typische Königinnenform. Von 10 in Völker (Apidea-Beuten) eingeweiselte Versuchsköniginnen flogen 7 erfolgreich zur Paarung aus und begannen mit der Eilage, von den Kontrollköniginnen waren es 6. Alle diese Königinnen erzeugten hunderte von Arbeiterinnen.

***Apis mellifera* / Embryo / Kerntransplantation / Klonierung / Cryokonservierung**

REFERENCES

[1] Ashburner M., Drosophila. A Laboratory Handbook, Cold Spring Harbor Laboratory Press, New York, 1989.

[2] DuPraw E.J., The honeybee embryo, in Wilt F.H., Wessels N.K. (Eds.), Methods in Developmental Biology, Crowell Press, New York, 1967, pp. 183–217.

[3] Fleig R., Sander K., Blastoderm development in honeybee embryogenesis as seen in the scanning electronmicroscope, Int. J. Invertebr. Reprod. Dev. 8 (1985) 279–286.

[4] Koeniger N., Hemmling C., Yoshida T., Drones as sons of drones in *Apis mellifera*, Apidologie 20 (1989) 391–394.

[5] Omholt SW., Ådnøy T., Effects of various breeding strategies on diploid drone frequency and quantitative traits in a honeybee population, Theor. Appl. Genet. 89 (1994) 687–692.

[6] Omholt S.W., Hagen A., Elmholdt O., Rishovd S., A laboratory hive for frequent collection of honeybee eggs, Apidologie 26 (1995) 297–304.

[7] Omholt S.W., Rishovd S., Hagen A., Elmholdt O., Dalsgard B., From S., Successful production of chimeric honeybee larvae, J. Exp. Zool. 272 (1995) 410–412.

[8] Rembold H., The role of determinator in caste formation in the honey bee, in: Lüscher M. (Ed.), Phase and Caste Determination in Insects, Pergamon Press, Oxford, 1976, pp. 21–34.

[9] Rembold H., Lackner B., Rearing of honeybee larvae in vitro: Effect of yeast extract on queen differentiation, J. Apic. Res. 20 (1981) 165–171.

[10] Ruttner F., Biogeography and Taxonomy of Honeybees, Springer, Berlin, 1988.

[11] Yu R., Hagen A., Omholt S.W., Cryopreservation of totipotent nuclei from honeybee (*Apis mellifera*) embryos by rapid freezing, Cryobiology 35 (1997) 41–45.