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Characteristics of the spermathecal contents of old and young honeybee queens

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ABSTRACT

Sperm are often stored, for a long time after mating, in females of various animal species. In case of the queen honeybee (*Apis mellifera*), sperm remain fertile for several years in the spermatheca. Little information is available regarding the effect of long-term storage of sperm on its fertility. To evaluate this, enzymes and/or sperm have been analysed from the spermatheca of 75 queens of various ages (0 year Y0, $n = 14$; one year Y1, $n = 14$; two years Y2, $n = 7$; virgin queen VQ, $n = 40$) and semen samples have been taken from 46 drones. The sperm from the spermatheca of older queens move more slowly ($F = 11.45$, $P < 0.0001$) and show different movement patterns ($Chi^2 = 90.0$, $P < 0.0001$) from those of the other groups. The spermatheca content of differently aged mated queens differ significantly with respect to the activities of lactate dehydrogenase ($F = 3.37$, $P < 0.05$), citrate synthase ($F = 6.24$, $P < 0.005$) and arginine kinase ($F = 9.44$, $P < 0.0006$). Glyceraldehyde 3-phosphate dehydrogenase ($F = 0.10$, $P = 0.91$) does not differ significantly. The results suggest considerable changes in the energy metabolic profile of the spermatheca tissue, of the sperm or of both during sperm storage.

1. Introduction

Artificial insemination is an important technique to improve and control animal breeding. Semen, however, exhibits a reduction in its fertility within hours or days, as has been shown for most species, and therefore cryopreservation protocols have been developed for the semen of man and several other animal species for its long-time storage over decades (Curry, 2000). However, semen of several species (e.g. turkey, boar and honeybee) have until recently been difficult to cryopreserve with sufficient quality.

In several species of insects, birds and reptiles, sperm can be stored naturally after mating for months or even years in a specific organ found in the mated females. Nevertheless, several studies have shown a decrease in sperm quality during storage, e.g. in the grasshopper *Chorthippus parallelus* Zett (Reinhardt et al., 1999), chicken (Nalbandov and Card, 1943), European hare *Lepus europaeus* (Stavy and Terkel, 1992), horse *Equus caballus* (Day, 1942), garter snake *Thamnophis sirtalis* (Rahn, 1940; Halpert et al., 1982; Birkhead, 1993), and turtles (Gist and Congdon, 1998; Pearse et al., 2001). Honeybee queens appear to be an ideal model organism for studying sperm storage in females because honeybees have the advantage of producing semen that survive for several years (Locke and Peng, 1993; Winston, 1987) and are suitable for management in apicultural research.

Little information about the mode of sperm conservation within the spermatheca of insects, including honeybee queens is available. Honeybee (*Apis mellifera*) queens mate early in life during the nuptial flight with a number of drones (Lobo and Kerr, 1993). The semen is stored in the spermatheca of the queens for the duration of their lives and kept alive and capable of fertilisation *for* up to five years (Taber, 1954; Verma, 1974; Weirich et al., 2002). The total number of spermatozoa extracted from the spermatheca of freshly mated queens varies between 1 and 8 million per queen (Koeniger and Koeniger, 2000; Cobey, 2007).

Honeybee spermatozoa measure 250–270 μm in length (Lino-Neto et al., 2000). The head consists of the anterior acrosome and the nucleus, which is 5 μm long, 0.5 μm wide and 0.3 μm thick (Lensky et al., 1979). Axoneme forms the central portion of the tail with nine single accessory microtubules most external, followed by the nine doublets and central pair (Rothschild, 1955). As in the other insect species (Baccetti et al., 1969), the tail of the honeybee spermatozoon contains two mitochondrial derivatives extending from the base of the nucleus nearly to the end of the tail (Rothschild, 1955).

Verma (1973, 1974, 1978a,b) has found that the concentrations of Na^+ and K^+ in the spermatheca fluid of the queen are important factors for the reversible suppression of sperm motility and longevity both in vivo and in vitro. Also the addition of exogenous amino acids (L-lysine, L-arginine, L-glutamic acid) and the enzyme

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catalase was found to allow a great number of respiratory active spermatozoa to survive in spermatheca for years (Verma, 1978c). Klenk et al. (2004) have demonstrated a pH value of 8.6 and a high protein concentration of 8.5–15.3 mg/ml in the spermathecal fluid; they have hypothesised that these values are important for the long-term storage of spermatozoa.

Weirich et al. (2002) have established that extracts of spermatheca of mated queens show remarkably high activities for enzymes: catalase (CAT), glutathione S-transferase (GST) and superoxide dismutase (SOD). They conclude that these spermathecal enzymes contribute to the protection of the spermatozoa from oxidative stress and thereby facilitate their long-term survival. These enzymes can increase sperm longevity by reducing the levels of damaging reactive oxygen species ROS (hydroxyl, hydroperoxyl radicals and hydrogen peroxide) (Pardini, 1995). In agreement, Collins et al. (2004) have found catalase transcripts particularly in the reproductive tissues and semen of male and female honeybee, thereby providing further evidence of anti-oxidative protection. Kraft et al. (1978) have performed a comparative analysis on sperm CO_2-production and found that respiratory glucose consumption of honeybee spermatozoa is relatively low compared with the sperm of other species. Nevertheless, the sperm retain their respiratory activity during storage, which presumably involves a high risk of oxidative damage (Weirich et al., 2002; Collins et al., 2004).

In this study, we provide further insight into some characteristics of sperm storage in honeybee spermatheca. Changes have been analysed during two years of storage in spermatheca with regard to sperm number, speed, and motility as well as the capacity of energy metabolism.

Energy metabolism is essential for all living cells and the intracellular pathways delivering ATP are adapted to the cell environment, its function, and the source of fuel that is available. Carbohydrates and fat are the two main fuels but others such as lactate or amino acids should not be neglected. The advantage of carbohydrates is that ATP can be produced in the absence of oxygen (anaerobic metabolism) and they can be stored intracellularly as the polymer glycogen. A second energy store is the phosphagen (phosphoarginine in insects). It is used for rapid ATP regeneration during activity or for the intracellular transport of energy-rich phosphate. An increase of the maximum activities indicates enhanced enzyme synthesis so that cells can adapt to sustained high energy demand, whereas a decrease of maximum activities is produced either by controlled enzyme degradation or by cell damage.

For honeybee spermatozoa, carbohydrates appear to be important for ATP production and the two long mitochondrial derivatives suggest aerobic metabolism (Blum and Taber, 1965). To examine whether the capacity of energy metabolism of sperm and spermatheca changes during the age of queens, we have analysed the maximum activities of enzymes involved in aerobic and anaerobic carbohydrate metabolism together with those of phosphagen kinase indicating the importance of an ATP-buffer or energy shuttle system.

2. Materials and methods

Naturally mated queens of known age were sampled from several beekeepers and different locations and kept in small mating bee nucs until the time of the experiment. In two sets of experiments during 2005 and 2006, semen from the spermatheca of each of 14 freshly mated (Y0), 14 one-year-old (Y1) and 7 two-year-old (Y2) queens were analysed. In addition in 2006, the content of the spermatheca of each of 20 one-week-old virgin queens (WVQ) and 20 one-month-old virgin queens (MVQ) and fresh semen (36 μl) collected from 46 mature drones were examined. Semen of drones was collected after Mackensen and Roberts (1948). Semen of drones and the spermatheca of unmated queens were investigated to determine whether the enzyme activities measured in spermathecal extracts of mated queens derived from the contained sperm or from the spermathecal tissue.

2.1. Preparation and extraction of spermatheca of mated queens

A glass Petri dish filled with bee's wax was used as a pinning out base for queen dissection (Dade, 1985). The head, wings and legs of the queens were removed; an incision along the length of the abdomen cuticle was made from dorsal to thorax with high quality micro-dissection scissors. A living female queen was pinned onto the glass Petri dish by using high grade stainless steal insect pins in her thorax; the cuticle was raised with a few pins, thereby exposing the internal organs of the queen and the spermatheca (Rothschild, 1955; Poole, 1970). Using fine point forceps, the spermatheca was removed and placed in a 1.5 ml micro-centrifuge tube and disrupted by mean of sonication. The content of each spermatheca (not exceeding 1 μl) was diluted with 250 μl of a solution containing 2.43 g Na_3-citrate·2-H_2O, 0.21 g $NaHCO_3$, 0.04 g KCl, 0.30 g sulphur acetamide, 0.30 g D-glucose and distilled water to 100 ml (Morse, 1994). The pH ranged between 7.5 and 8.4. Fresh semen samples (on average 4 μl each) were equally diluted for comparisons with spermathecal content.

2.2. Analysis of sperm number and motility

Sperm number and motility were analysed in diluted sperm suspensions obtained from the spermatheca of mated queens. Sperm movement patterns were observed in 2.3 μl sperm suspensions in 20 micron analysis chamber slides (Leja, 2153 GN Nieuw-Vennep, The Netherlands) and analysed by a computer-assisted semen analysis (CASA) system (ESHRE Andrology Special Interest Group, 1998; Kime et al., 1996). Because of the marginal differentiation between head and tail in honeybee sperm, the CASA system was less efficient in measuring the speed of the sperm. Multiple photomicrographic exposure and video-micrographic techniques for spermatozoa track analysis (Boyer et al., 1989) and a computer equipped with imaging software (Adobe Photoshop and Quik Time) were used to measure the speed of individual sperm. Here, the head of a sperm was marked in a 1 s interval and the distance that it covered was calculated by the software. Five randomly selected sperm per queen were analysed.

From the original suspension, a 25 μl sample was diluted with 25 μl distilled water to count the spermatozoa per spermatheca. From the final volume, 20 μl (10 μl in each counting chamber) were used to count the spermatozoa per spermatheca by using a standard hemacytometer counter chamber slide under a light microscope (Shiran et al., 1995; Lu et al., 2007).

2.3. Enzyme analysis

Maximum activities of lactate dehydrogenase (LDH, EC 1.1.1.27), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) and arginine kinase (ArgK, EC 2.7.3.3) were spectrophotometrically analysed by using NAD(P)H as indicators at 340 nm according to Bergmeyer (1983). For citrate synthase (CS, EC 4.1.3.7), the absorption of the 5,5-dithionitrobenzene (DTNB)–CoA complex was detected at 413 nm. The assays were performed at 25 °C with two different sample volumes to examine the correlation between enzyme concentration and activity. Total volumes of all tests were 500 μl and the assays were started by addition of the substrate. Lactate dehydrogenase assay contained