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BIOCHEMICAL CHANGES DURING HONEY BEE FLIGHT MUSCLE DEVELOPMENT

Though the flight muscles of the honey bee are formed mainly during the pupal period (Gertel 1930)) several changes in ultra structure occur after emerging of the imago. Herold (1965) showed that the sizes of the sarcomeres during honey bee flight muscle development increase up to a twelvefold in volume from the newly emerged adult period to adult maturation (Fig.1). This phenomenon is also well documented in other holometabolic insects (Rockstein and Miquel (1973)). The structure of myofibrils and mitochondria in the newly emerged imago show not the regular pattern of the mature adult. In the old imagines often fusions of different mitochondria can be observed and several mitochondria may be included in one ecto-mitochondrial membrane. Beside these histological changes there were also changes in metabolic balance, indicated by the reddish coloration of fully functional muscle tissue. The flight muscle of emerging bees have a white colour similar to the muscle tissue in the legs and mandibles in other insects (Sacktor 1974). This tissue is discussed as to be better adapted to anaerobic periods whereas the flight muscle of honey bees shows an extremely high aerobic glycolytic flux. Herold and Borei (1963) showed that the increased red coloration of developing flight muscle is correlated to an increase of various cytochrome components. Beside cytochrome b, all other cytochromes showed a higher concentration in older bees. The glycolytic capacity of the honey bee flight muscle was demonstrated by Hersch et al. (1976a, 1978b). These authors tested the enzymes hexokinase (HK), glycerol-3-phosphate-dehydrogenase (G3PDH) and lactate-dehydrogenase (LDH). Whereas the aerobic glycolytic enzymes (HK, G3PDH) raised in activity correlated to the age, LDH as an indicator for anaerobic glycolysis remained at a very low level. The high HK-activity implies that glucose rather than glycogen
MATERIALS AND METHODS

Sealed worker and drone brood of Apis mellifera was kept in an incubator (34°C, 60% r.h.) until the imagines hatched. Newly emerged workers and drones were marked individually to determine the exact age later on. The flight muscles were excised carefully at 5°C. Samples of 200 mg wet weight were homogenized in 2 ml isotonic sucrose solution using a Potter homogenizer with a teflon pestle. The homogenate was centrifuged in a 0.25 M sucrose solution analogous to that of Blume (1979) (Fig. 2). Since specific mitochondrial enzymes were tested, there was no need for a high grade purification of the washed mitochondrial fraction by gradient centrifugation (Oswalai and Rembold (1968)). Adhering lysosomes and peroxysomes which are only infrequently encountered in flight muscle tissue (Rockstein and Miguel (1973)) are not separated quantitatively by this method. They do not interfere with the experiment because they contain mostly proteolytic enzymes which were not tested in this assay. The mitochondrial fraction was resuspended in 100 μl of 0.25 M sucrose solution after centrifugation.

Malate dehydrogenase (MDH) was determined according to Bergmeyer and Bernt (1974). The buffer substrate solution (1.6 ml of: 92 mM phosphate buffer (pH 7.4); 39 mM aspartate; 1 mM ketoglutarate; 0.18 mM NADH) was incubated for 5 minutes at 25°C before the addition of the test sample (5 μl mitochondrial suspension). The mean absorbance change per minute at 340nm (1 cm light path) was taken as the measure of activity.

Isocitrate dehydrogenase (ICDH) activity was measured by the method of Wolfson and Williams-Asman (1957). A 25 μl sample was added to 1.25 ml buffer-substrate solution (100 mM triethanol amine buffer (pH 7.5); 4.6 mM isocitrate, 52 mM NaCl) and incubated for at least 5 min. at 25°C. After the addition of 50 μl 9.1 mM MnSO₄ absorbance change per minute at 340nm (1 cm light path) was determined.

Glutamate pyruvate transaminase (GPT) activity was assayed according to the Deutsche Gesellschaft für klinische Chemie (1970). A 25 μl sample was added to 1.55 ml buffer-substrate solution (80 mM phosphate buffer (pH 7.4); 800 mM L-alanine, 0.188 mM NADH, 1.20 U/ml LDH, 18 mM α-oxoglutarate) and assayed at 340 nm, 25°C and 1 cm light path.

Glutamate oxaloacetate transaminase (GOT) was measured by the method of Karmen (1955). The sample (25 μl) was added to 1.55 ml buffer-substrate solution (80 mM phosphate buffer (pH 7.4); 32.5 mM L-aspartate, 0.18 mM NADH, 0.06 NADPH, 1.2 U/ml LDH, 6.7 mM α-oxoglutarate). Absorbance change was assayed at 25°C, 340 nm and 1 cm light path.

Aldolase activity was determined according to Beisenherz et al. (1953). A 5 μl sample was added to 1.25 ml buffer-substrate solution (51 mM colloïde buffer (pH 7.5),

is the major energy source in the honey bee flight muscle. As mitochondria obviously are very strongly involved in the cytological changes of the flight muscle it seemed worth while to have a closer view on the metabolical pathways and enzymes which are located on the sarcosomes.
To perform isoelectric focussing the flight muscles were homogenised as described above and centrifuged at 600g twice. 6ul of the supernatant was applied to the polyacrylamide gel (Servalyt Precote® pH 3-10, 100µm). The electrode fluids were composed as follows:

Anode: 0.83 g L-aspartic acid, 0.92 g L-glutamic acid dissolved in 250 ml water.

Cathode: 30 ml ethylene diamine, 1.09 g L-arginine free base, 0.91 g L-lysine free base filled up with water to 250 ml.

Isoelectric focussing was performed at 5 mA, 2.25 W, 1.2 kV for 120 min. Proteins were stained with Serva blue W (C.1. Acid blue 15) in aqueous solution after fixing the proteins with trichloroacetic acid (TCA 20%). The gels were densitometrically analysed.

RESULTS

MDH catalyses the reduction of malic acid to oxaloacetic acid and thus is an important enzyme in the Krebs cycle (Fig.3). Just after emergence in both, drones and workers, MDH showed only very low activities. Figure 4 shows the relative activities (maximum activity=100%) at the according ages. Though the absolute activities are higher in the workers (max. activity Q = 3 U/mg protein; max. activity Q = 1.5 U/mg protein) the drones reached their maximum MDH activity earlier than the workers. At the fourth day after hatching 100% activity is reached and it remained at this level for the whole testing period. The workers reached their maximum after 9 to 10 days.

The same phenomenon was found for the ICDH another enzyme of the Krebs cycle (Fig.5). In the workers a plateau of enzyme activity is reached after 9 days (Fig.6). As both enzymes of the citric cycle system increase in activity, the catabolic importance of this pathway seems to grow in the aging bees.

![Diagram of enzyme reactions]

**Fig.3** MDH catalyses the oxidation of malic acid to oxalacetic acid in the Krebs cycle.

0.27mM monoiodoacetate, 2.7mM fructose-1,6-diphosphate
0.22 mM NADH, 326 µM/ml GDH, 4.35 µM/ml TIM, 616 µM/ml LDH incubated for 5 min at 35°C. Absorbance was measured and after another 20 min of incubation extinction was determined again. The absorbance change indicated the enzyme activity in the sample (340 nm, 1 cm light path).

Protein content of the sample was tested with the Bloor method (5ul sample in: 0.1 N NaOH, 16 mM potassium sodium tartrate, 15mM KJ, 6mM CuSO4) at λ9 546nm and 1 cm light path.
The enzymes of the protein metabolism (GOT, GPT) were tested as well. In contrast to the citric cycle enzymes a decrease in activity was found in maturing bees (Fig. 7). Again the fourth or ninth day respectively seem to be the limit for activity changes. After this the enzyme activities remained rather constant. The activity changes observed in the workers are stronger than those observed in the drones.

This obvious reduction of protein metabolism agreed quite well with the data from the isoelectric focusing (Fig. 8). With an increasing age the protein pattern was reduced. Four protein bands disappeared successively when the bees became older (see arrows in figure 8). There were vast changes in quantitative protein content of the according bands in the beginning of imaginal life. At the 14th day the pattern remains constant till the end of the testing period (30 days). Thus it seems that protein metabolism becomes less active in aging bees.

The aldolase activity (Fig. 9) shows comparable results as obtained for the enzymes MDH and ICDH (Fig. 10). After low activities in the young bees it increased rather quickly to a maximum at the 8th day. After this the activity reduced to about 50% and remained on this level.

**DISCUSSION**

Catabolic pathways in insect flight muscle seem to be very species specific as Beenakkers et al. (1975) showed in a comparative study with Calliphora erythrocephala, Locusta migratoria and Philosoma cynthia. Mainly three different physiological types of insect flight muscle can be distinguished:

1) lipid utilizers
2) carbohydrate utilizers
3) combination utilizers

The honey bee definitely belongs to the carbohydrate utilizers as shown by Beenakkers (1969). The importance of the glycolysis was shown by Hersch et al. (1978a, 1978b) and by the data obtained for aldolase in this paper. We also could show that the Krebs cycle enzymes MDH and ICDH in flight muscle mitochondria are highly active in
mature and fully operating muscle tissue. In workers as well as in drones these enzymes show their maximum activity level when the age of active flying is reached. As drones perform their first flight earlier than the workers, the differences in MDR activity in both sexes seems to be plausible. This agrees quite well with data of Balboni (1967) who showed that the citric cycle rather than the a-glycerophosphate oxidase system is necessary for sustained flight in honey bees. Also Maruyama and Moriwaki (1957) who tested homogenized flight muscle tissue by means of the Warburg technique, could find an increase in catabolic speed in old bees (10 days after emerging) compared to the young ones (1 day old). Maruyama (1974) suggested that the ATP supplying system rather than the ATP utilizing system is rate limiting for the flight of honey bees. ATPase shows the maximal activity in late pupal stages and this activity level is maintained throughout the imaginal life.

Fig. 6  ICDH activities in mitochondria of workers (n=20 for each mean, enzyme activity in % of maximum = 148 U/g protein).

Fig. 7  a Transamination of glutamic acid. R* is CH3 in case of GPT R* is CH3,COOH in case of GOT. b GOT activities in mitochondria of workers and drones in % of maximum (O = 92 U/g; D = 55 U/g). c GPT activities in drone mitochondria in % of maximum (98.3 U/g) (n=20 for each mean).
is obtained by the isoelectric focusing. In the early stages of adult life the protein pattern is much more complex and quantitative changes occur more often and vaster in young bees than in old ones. This phenomenon was also observed by Engels and Fahrenhorst (1973). They separated the proteins in haemolymph samples of bees by electrophoretic methods and reported a rather constant protein pattern in old bees. In young bees they also could find substantial quantitative and qualitative changes during development.

Fig. 8 Protein pattern of flight muscle homogenate obtained by isoelectric focusing. The arrows indicate the missing band at the according age (n=30 for each mean, protein content in % of total).

The high activities of the transaminases in mitochondria of young workers and drones indicate a large protein metabolism in this period of life. This could be a sign of participation of amino acids in the catabolic pathway as demonstrated for Phormia regina and other insects (Sacktor and Childress (1967), Stevenson (1968), De Kort et al. (1973) and others). On the other hand this seems to be rather unlikely in this case of the honeybee, as there is no need for a large energy supply of the flight muscle as long as the bee does not fly. It might be more probable to interpret the high transaminase activities as a sign of increased protein synthesis, which may be necessary to achieve the complex changes in sarcosome structure of the developing flight muscle. This hypothesis is confirmed by the data which

Fig. 9 Aldolase splits fructose-1,6-diphosphate in dihydroxyacetone phosphate and D-glycerinaldehyde-3-phosphate in the glycolysis.
The reduction of the aldolase activity may represent a well known phenomenon in the aging of insects. Rockstein and Miguel (1973) showed comparable results for the enzymes ATP:arginine phosphotransferase, α-glycerophosphate dehydrogenase and ATPase in flight muscles of the male house fly. Those enzymes raised in activity after emergence very quickly to a maximum of 100% at the 2nd or 5th day respectively. After this the enzyme activity dropped down to a 50% level which is discussed as to be an aging effect.

Thus it may be concluded that glycolysis as well as the Krebs cycle and the cytochrome system are controlling the flight capabilities of the developing honeybee. As soon as these pathways reach their maximum activity sustained flight can be performed. Once the bees are active fliers the activities of transaminases and the complexity of the protein pattern reduce to a constant level. Obviously mitochondrial enzymes seem to play a very important role in the metabolism of the flight muscle in both, nonflying and flying bees.

**ABSTRACT**

Several mitochondrial enzymes of honey bee flight muscles of workers and drones were tested. The enzymes of the Krebs cycle (MDH, ICDH) showed an increase in activity in aging bees. The transaminase activities and the complexity of protein pattern (isoelectric focussing) decreased in maturing bees, indicating a reduced protein metabolism in active flying workers and drones.

**REFERENCES**


**Fig. 10** Aldolase activity in flight muscle of worker bees in % of maximal activity (400 U/g, n=20 for each mean).