Haemolymph sugar homeostasis and the control of the proventriculus in the honeybee (*Apis mellifera carnica* L.)

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

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aus Neumünster

Würzburg 2001

Eingereicht am:

Mitglieder der Promotionskommission:

Vorsitzender:

Gutachter:

Gutachter:

Tag des Promotionskolloquiums:_____

Doktorurkunde ausgehändigt am:_____

For my child minder

Rosi.

and everybody else who enables mothers to continue working.

Physiology is not only a description of function; it also asks why and how. To understand how an animal functions, it is necessary to be familiar both with its structure and with some elementary physics and chemistry.

Knut Schmidt-Nielsen, "Animal Physiology"

1. GENERAL INTRODUCTION

In social insects, feeding benefits the colony as well as the individual. For most social insects it is true that their larvae and nestmates have to be provided for by workers that forage at a distance to bring back their food. The main energy source for social insects are carbohydrates. While isoptera (termites) meet their carbohydrate needs via cellulose ingestion, social hymenopterans (ants, bees and wasps) feed mainly on liquid carbohydrates, which are transported to the colony in the crop. But the individual forager also has to support its own metabolic needs from the crop content. As no digestion occurs in the crop, some of the crop content has got to be transported into the midgut, where the digestion takes place. This transport is regulated by the proventriculus, a valve which links these two compartments of the gut. Food that has passed this valve is lost for the colony, so that it has to be assumed that selective pressure has resulted in a precise regulation of the proventriculus activity. The aim of the present study was to investigate the factors involved in the regulation of the proventriculus activity in honeybees.

In honeybees, the passage of liquid food from crop to midgut was observed to depend on the concentration of the ingested sugar solution and on the locomotory activity of the individuals (Schreiner, 1952). Núñez et al. (1974) suggested haemolymph sugar titers, especially that of trehalose, as controlling variables for the proventriculus activity. More recently, Crailsheim (1988c) suggested an increase in haemolymph osmolarity as the factor controlling crop emptying rates in honeybees. The results of a previous study suggest the haemolymph sugar titers, rather than haemolymph osmolarity, as the factor controlling the activity of the proventriculus (Roces and Blatt, 1999).

As changing experimental conditions are very likely to influence the activity of the animals, and as in turn the solution transport rate from crop to rectum is surely dependent on the metabolic rate, a basic problem of all previous investigations was that the metabolic rate of

the investigated animals had never been controlled. Therefore in this thesis the metabolic rate of every investigated individual was measured.

As the studies mentioned above have suggested the haemolymph to be a decisive factor in the control of the proventriculus, chapter 3.1. deals with the dependence of haemolymph sugar titers on metabolic rate and with the question whether haemolymph sugar titers are regulated at all.

The aim of the next chapter (3.2.) was to analyse the temporal dynamics of the proventriculus activity and to analyse to what extent the input variables food quality (concentration, molarity and viscosity of the fed solution) and food quantity have an impact on the regulation of the proventriculus.

Chapter 3.3. focuses on the effects of internal state variables (haemolymph osmolarity, haemolymph sugar titer and foraging motivation) on the proventriculus activity.

The focus of the thesis then moves to an investigation of whether regulated haemolymph sugar titers are also observed in honeybee foragers collecting nectar from natural food sources, and whether the haemolymph sugars correspond with the predictions based on the results obtained with trained honeybees foraging at artificial food sources (chapter 3.4). The second part of this chapter deals with the question whether sucrose occurs in the honeybee haemolymph or if it represents an experimental contamination.

1.1. Structure of the honeybee gut

The central part of the honeybee gut is located in the gaster and connected to the mouth via the long oesophagus (Fig. 1). There are three main regions to the gut, with sphincters (valves) controlling food/fluid movements between them. The foregut (pharynx, oesophagus, crop and proventriculus) is concerned with ingestion, storage and transport of the food to the next region, the midgut, where most of the digestion and absorption takes place. The material remaining in the gut lumen together with urine from the Malpighian tubules then enters the hindgut. Water and feces are stored in the rectum, until the bees can fly from the hive and defecate.

The honeybees transport the nectar in the crop, which is small, when empty, but which can expand to a huge balloon occupying much of the gaster cavity, when full (Snodgrass, 1956). At an unladen body weight of only 60 to 80 mg, bees can store about 60µl in the crop. The extreme elasticity of the crop results from the highly folded epithelium (Brosch and Schneider, 1985). The honeybee crop is incapable of absorption because of the cuticular lining

(Pasedach-Poeverlein, 1940), so that no substances are lost while the food is stored in this organ. The crop contents can be regurgitated by a contraction of surrounding muscles.

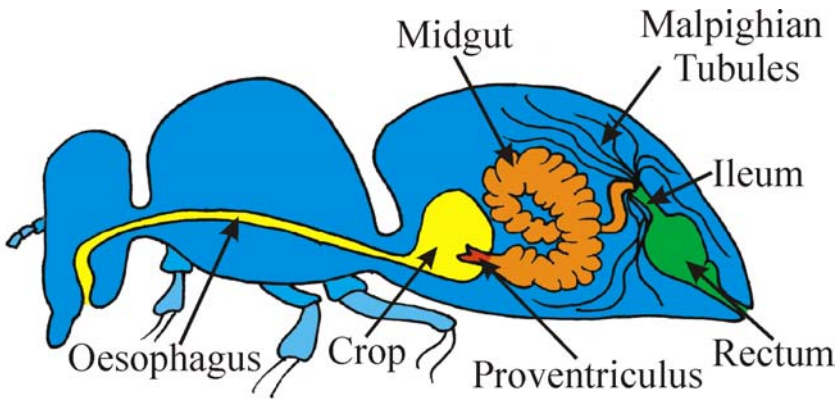

Fig.1. Scheme showing the structure of the honeybee gut.

1.2. Structure and function of the honeybee proventriculus

In the adult honeybee the proventriculus is modified into a morphologically and functionally unique structure. Its anterior portion projects like a plug into the crop; it is a conical-shaped structure measuring 0.7mm in diameter x 0.5mm in height and bearing 4 triangular-shaped lips (Fig. 2). The four lips can be closed tightly by the contraction of circular muscles (Peng and Marston, 1986). The inner margin of the anterior lip bears two types of hairs: long, filiform-hairs which form a comb along the margins, and spine-like short hairs which are located externally to the combs of filiform-hairs (Peng and Marston, 1986).

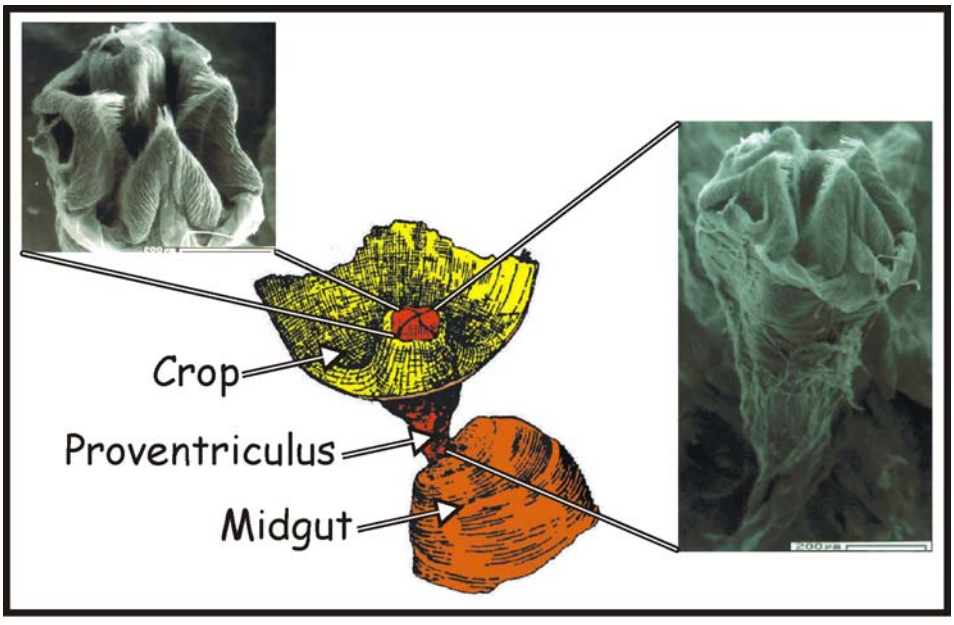

Fig. 2. Scheme and scanning electrone micrographs showing the proventriculus of the honeybee.

The proventriculus regulates the food transport from crop to midgut (Bailey, 1952) and prevents backflow of food from the midgut (Snodgrass, 1956). It also functions as a filtering apparatus for extracting pollen (Bailey, 1952; Peng and Marston, 1986). By video-recording the proventriculus action it was observed that the lips of the proventriculus were continuously making snapping and catching movements. Less often, but still regularly the proventriculus was making gulping movements, so that one gets the impression that these are the moments, when solution was swallowed into the midgut (own observations). Peng and Marston (1986) found that pollen were caught by the opening action of the lips and that they were then packed underneath the comb of hairs.

1.3. Sucrose digestion

The ingested sucrose solution is stored in the crop, where no digestion and no absorption occurs (Maddrell and Gardiner, 1980). When solution is released into the midgut, sucrose is hydrolised into glucose and fructose (Fig. 3). Though the largest part of sucrose is hydrolised in the midgut, it can cross the midgut wall (Turunen, 1985). The sugars cross the gut wall by facilitated diffusion, following the concentration gradient (Crailsheim, 1988b; Turunen, 1985). Though bees possess relatively high glucose and fructose haemolymph titers, the concentration gradient is maintained by the conversion of glucose into the non-diffusive trehalose, thus effectively trapping the carbohydrate and promoting uptake from the gut in the absence an active transport system. The conversion of glucose into trehalose takes place in the fat body. Trehalose synthesis occurs via UDP-glucose and trehalose-6-phosphate. The process requires energy in the form of ATP and UTP. Trehalose is released from the fat body into the haemolymph, where it is the major haemolymph sugar. Using the non-reducing disaccharide trehalose for this function has two advantages, first it does not react with some amino acids and proteins as glucose does and secondly the osmotic effect is only half that produced by an equivalent amount of glucose (Becker, et al., 1996).

Fructose is converted into trehalose only via first being converted into glucose. The absorbed glucose and fructose are converted into trehalose within 2min (Gmeinbauer and Crailsheim, 1993). In the mitochondria trehalose is hydrolised into glucose, which then is metabolised to water and CO_2 (Brandt and Huber, 1979; Friedmann, 1978). While the CO_2 leaves the animal via the trachea, the water is filtered out of the haemolymph through the malpighian tubules, from where it enters the rectum. (Crailsheim, 1988a) found that the diffusion of glucose and fructose from the midgut into the haemolymph is not the limiting factor for the sugar intake, but that it is the solution transport rate from crop to midgut. Though it can not be ex-

cluded that some of the glucose is used to be converted into glycogen, this can be neglected, as forgers maintain only very small amounts of body reserves (Beutler, 1937; Neukirch, 1982).

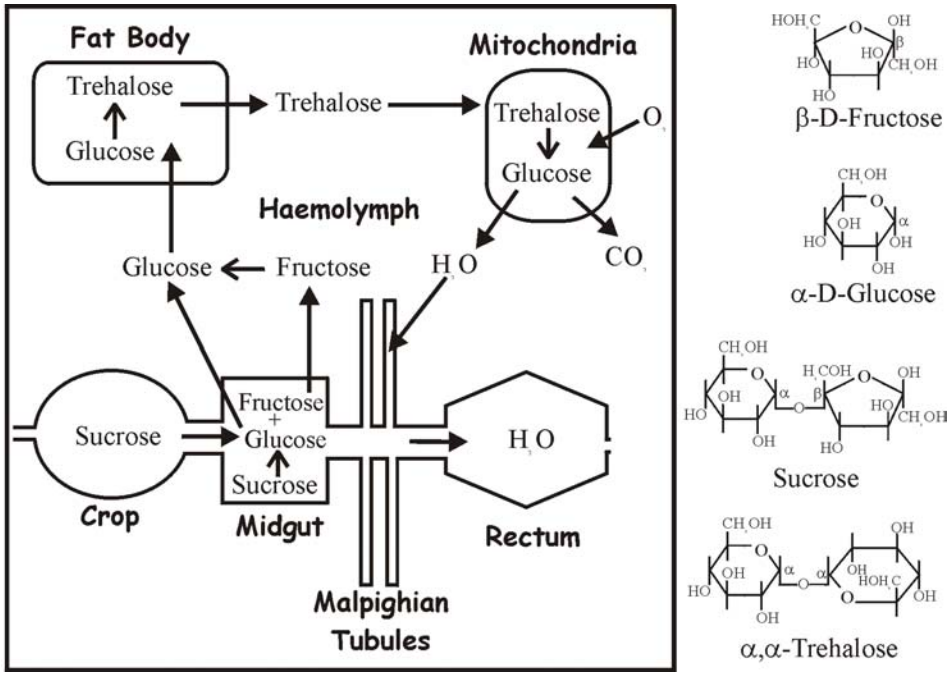

Fig. 3. A simplified sheme illustrating the sucrose digestion in the honeybee and the Haworth-ring forms of the involved sugars.

2. MATERIALS AND METHODS

2.1. Experiments with trained honeybees

2.1.1. Standardisation

A queenright colony of honeybees *Apis mellifera carnica* (Pollm.) was kept in a two-frame observation hive (Fig. 4A) in the vicinity of the bee station of the University of Würzburg, Germany. Experiments were carried out from the beginning of June to the end of August in the years 1996-1998.

It is well known that foragers react to increasing reward (sugar solution) concentrations with increasing flight speed (von Frisch and Lindauer, 1955; Gmeinbauer and Crailsheim, 1993), so that different feeding conditions have an impact on the foraging motivation of the bees. Therefore, an experimental design was used that guaranteed relatively undisturbed foraging behaviour of the bees. They were trained to fly 80m from the hive to a feeding station located in the laboratory. The bees reached the feeding station through a 12cmx15 cm hole in a window, which led into a wooden, ”L”-shaped tunnel (110 cm long, 12 cm wide, 15 cm high) with a Plexiglas top. Green and yellow reference marks were drawn on the bottom and on both sides of the tunnel (Fig. 4B). Trained bees flew directly to the end of the ”L”, where the feeder was located. The feeder consisted of a glass container filled with a polystyrene block in which a tiny glass cup was located.

During the training stage, all incoming foragers were allowed to feed *ad libitum* (Fig. 4C). During the experiments, only one bee was allowed to enter the tunnel at a time. It found 15, 30 or 50µl of the same sucrose solution used in the training stage (Fig. 4D;E). As this quantity does not represent the maximal crop load (60 - 70 µl), the bee always collected all the solution provided. While the bee imbibed the sugar solution (approximately 20 - 40 s), the feeder with the bee was carefully placed into a respirometric chamber (height 44 mm, diameter 61 mm, volume 128 cm^3) in which it walked freely after feeding and did not try to fly. Only bees that did not interrupt feeding while being placed into the respirometric chamber were used in the experiments(Fig. 4F).

The concentrations of the fed solutions were reported as percentage sucrose equivalents (g solute/100 g solution) (following Bolton *et al*., 1979). The concentrations used during the experiments, 7.5, 15, 30 and 50% sucrose (w/w), are in the range of nectar sugar concentrations that bees encounter naturally (Baker and Baker, 1978).

2.1.2. Gas exchange measurements

After food collection, the CO_2 production of each bee was measured over different times, depending on the experiments. These times were chosen on the basis of preliminary experiments, so that at the end of the CO_2 measurements the bees would still have sugar solution in their crop and therefore any changes in haemolymph sugar titers were not the result of exhaustion of the crop contents during the measurement period.

Open-flow respirometry was used to measure CO_2 production of bees after feeding. CO_2-free air was drawn through the respirometric chamber at a flow rate of 300 $mlmin^{-1}$, which was controlled by a mass-flow controller. The high-resolution respirometry system used (Sable System TR-3, resolution 0.01 ppm CO_2), including temperature control and correction to S.T.P.D. conditions, has been described elsewhere (Lighton, 1990). To obtain measurements over a wide range of metabolic rates, the respirometric chamber was placed in a water bath with temperatures ranging from 10 to 39°C. Preliminary experiments showed that foraging bees tried to maintain high metabolic rates after feeding even at relatively low temperatures. Metabolic rates were expressed in ml CO_2h^{-1} per bee, not as mass-specific rates, to allow comparisons, because the measurements of sugar transport rates through the proventriculus (see below) were calculated for the whole animal. The range of body masses of unfed bees varied between 71 and 85 mg (mean ± S.E. = 79.1 ± 4.9 mg, N= 22).

2.1.3. Injections

For some investigations it was necessary to inject the bees. In order to decide whether the bees should be anaesthetised during injections and whether the injected substances should be dissolved in water or in Ringer solution, bees that were fed 30µl 30% sucrose solution and remained in the gas exchange chamber for 30min were divided into two groups. The bees of one group were anaesthetised with CO_2 after 15 min, while the other group went through the experiments without anaesthetisation. Each group was subdivided into 4 sets of 8 bees, which were used for the Ringer/water-experiments:

1. just fed or just anaesthetised after 15min, dependent on the group
2. merely punctured into the neck after 15min
3. injected with 1µl water after 15min
4. injected with 1µl Ringer after 15min

As the relation between crop emptying rates and metabolic rates was equal for all examined groups, it was decided to anaesthetise the bees before injection. This made the handling of the bees much easier so that injections could be applied with more caution. No differences

concerning crop emptying rates were found regardless of whether the bees were injected with water or Ringer. As measurements of the ion compositions showed that the bee haemolymph contained ions in the same range as found for other insects (own measurements), it was decided to dissolve the sugars in Ringer.

Three different places for injections have been described in literature: between the tergits of the gaster (Bounias and Morgan, 1985; Crailsheim, 1988a; Loh and Heran, 1970; Van der Horst et al., 1978), through the leg base into the thorax (Woodring et al., 1993); and into the neck membrane (Roces and Blatt, 1999). Injections into the gaster have the advantage that injected solutions are most thoroughly diluted in this largest compartment. Injections into the gaster did, however, produce some experimental difficulties. Firstly, one could never be sure not to have injured the crop, especially when the bees had collected a large amount of solution. Secondly, injections into the gaster sometimes caused bleeding immediately after injection, so that it was not clear how much of the injected solution had really entered into the haemolymph circulation. For injections into the leg base, rather high pressure had to be applied, so that the bees sometimes regurgitated their crop content. As opposed to these two methods, injections into the neck membrane did not cause any of the above problems, if the injections were performed ventrally and flatly under the membrane. However, to investigate if the molarity changes caused by the injections led to different crop emptying rates when bees are injected into the large gaster or into the neck, in one experiment trehalose solution were injected into the gaster or into the neck respectively.

Consequently, bees standardised as described above were handled in the following way during injection experiments: Before injections, the gas exchange chamber was opened and each bee was made to run into a snap lid glass. CO_2 flowed into the glass through a hole in the lid for about 10 sec. The bee was then held at the wings, the protruding head of the anaesthetised individual was deflected ventrally and an amount of 1µl of the respective solution was very carefully injected through the dorsal neck membrane with a Hamilton syringe (needle gauge 33 µm) (Fig. 4J). Bleeding was almost never observed, and if it occurred, the bee was not used for the experiments. After injection the bee was put back to the gas exchange chamber and their CO_2 production was measured until dissection. Normally the whole procedure did not take longer than 30sec. Only bees which recovered during 1min and showed a normal gas exchange pattern afterwards were used for the experiments. Solutions of metabolisable sugars (1M Trehalose, 1 or 2M Glucose and 1 or 2M Fructose) or non-metabolisable sugars (1M Sorbose) were injected either 5, 15 or 20min after feeding. Ringer solution (9.3g NaCl, 0.5g KCl, 1.2g $CaCl_2 \cdot 2H_2O$, 0.8g $MgCl \cdot 6H_2O$ per litre distilled water) after Woodring et al.

(1994) was used as solvent. Furthermore, two groups of control bees were analysed: uninjected bees and bees injected with 1µl of Ringer solution.

2.1.4. Measurement of crop emptying and sugar transport rates

As honeybee foragers flying to a familiar feeding place carry only as much sugar solution in the crop as they need for their flight (Beutler, 1950; Sacktor, 1970; Brandstetter *et al*., 1988), training ensured that both the crop and rectum of workers arriving at the feeding station were empty. This was confirmed by dissections of control bees arriving at the feeder. Since bees were fed with a known quantity of sucrose solution, it was possible to determine the flow rate through the proventriculus by measuring the amount of solution in both the crop and rectum after specific time intervals. Since the concentration of the sucrose solution was known, the sugar transport rates through the proventriculus could then be calculated.

After gas exchange measurements the bee was gently caged and anaesthetised with CO_2 until the proboscis was extended, i.e. the individual was completely anaesthetised but did not regurgitate its crop contents. This procedure took less than 10 s. To measure the amounts of fluid contained in both the crop and rectum, the bee was fixed ventrally onto a wax plate after it had been anaesthetised, its abdomen was dissected and the haemolymph was absorbed with a piece of filter paper. The crop was carefully pulled out of the intestine with tweezers. The crop contents were squeezed out by pressing the crop against pre-weighed filter paper, so that the crop tissue remained between the tweezers. The moistened paper was put into a small pre-weighed vial and weighed on a microbalance (Mettler UMT5) to the nearest 0.001 mg (Fig. 4G). Crop content mass (in mg) was converted into a volume by dividing by the density of the fed sugar solution. To measure the rectum fluid content, an incision was made in the rectum wall, and the fluid was absorbed with pre-weighed filter paper. Again, the moistened paper was put into a small pre-weighed vial and weighed. Since the rectal fluid contained no sugars, its density was assumed to be 1 g/ml. Measurements of rectal fluid production were observed to correspond well with crop-emptying rates, thus providing a control for the fluid volume measurements and confirming that no changes in haemolymph volume that would affect measurements of haemolymph sugar titers occurred during the experiments (see also Roces and Blatt, 1999). The whole dissection procedure from the beginning of anaesthesia lasted less than 3min.

2.1.5. Haemolymph sampling and determination of sugar titers

To obtain a sample of haemolymph, the protruding head of the anaesthetised bee was deflected ventrally, the exposed dorsal neck membrane was punctured with a pin and 0.5 µl of haemolymph was collected with a microcapillary (Fig. 4H). Immediately after collection, each single haemolymph sample was placed in 400 µl of distilled water and kept at -20°C until analysis.

High-performance liquid chromatography (HPLC) was used to measure trehalose, glucose and fructose concentrations. Sugars were separated on a carbopac PA1 column at a flow rate of 0.9 ml/min. The HPLC running buffer consisted of 80 mM NaOH. Sugar concentrations were determined by isocratic ion chromatography with pulsed amperometric detection (4500i, Dionex, Idstein, Germany). The system was calibrated after every fourth sample with a standard solution containing 50 µmol/l each of trehalose, glucose, fructose and sucrose. Measurements were performed with a sensitivity of 3 µC. The detection limit for measurements was 5 µmol/l. Chromatograms were processed with Winpeak software (Chromatography Data System, Biotronik, Maintal, Germany). Results were converted into mg/ml.

2.2. Haemolymph sampling and determination of nectar concentrations for foragers returning from natural food sources

A queenright colony of the honeybees (*Apis mellifera carnica*) was kept in a two-frame observation hive in the vicinity of the bee station of the University of Würzburg, Germany. In order to test whether there is a correlation between both amount and concentration of the collected nectar and haemolymph sugar concentrations, bees returning from foraging trips were caught at the hive entrance. To ensure that the bees were disturbed as little as possible while being caught, a 15cm long passage was attached to the hive entrance so that individual foragers could be collected by placing a snap lid glass against the passage opening, which the bees entered voluntarily. The caught bees were immediately anaesthetised with carbon dioxide, after which the bees were forced to regurgitate the crop contents onto a hand refractometer by slightly pressing their abdomen. The sugar concentrations of the crop contents were measured as sucrose equivalents and reported as percentages (g solute/100g solution) following the convention of [Bolton, 1979 #221]. The amount of the crop contents were qualitatively estimated as small, medium or large. After this, haemolymph sugar samples were taken from every investigated individual as well. To avoid delays and perturbations, the whole procedure was carried out directly at the hive. Investigations were carried out in the European summer, from beginning of May to end of August.

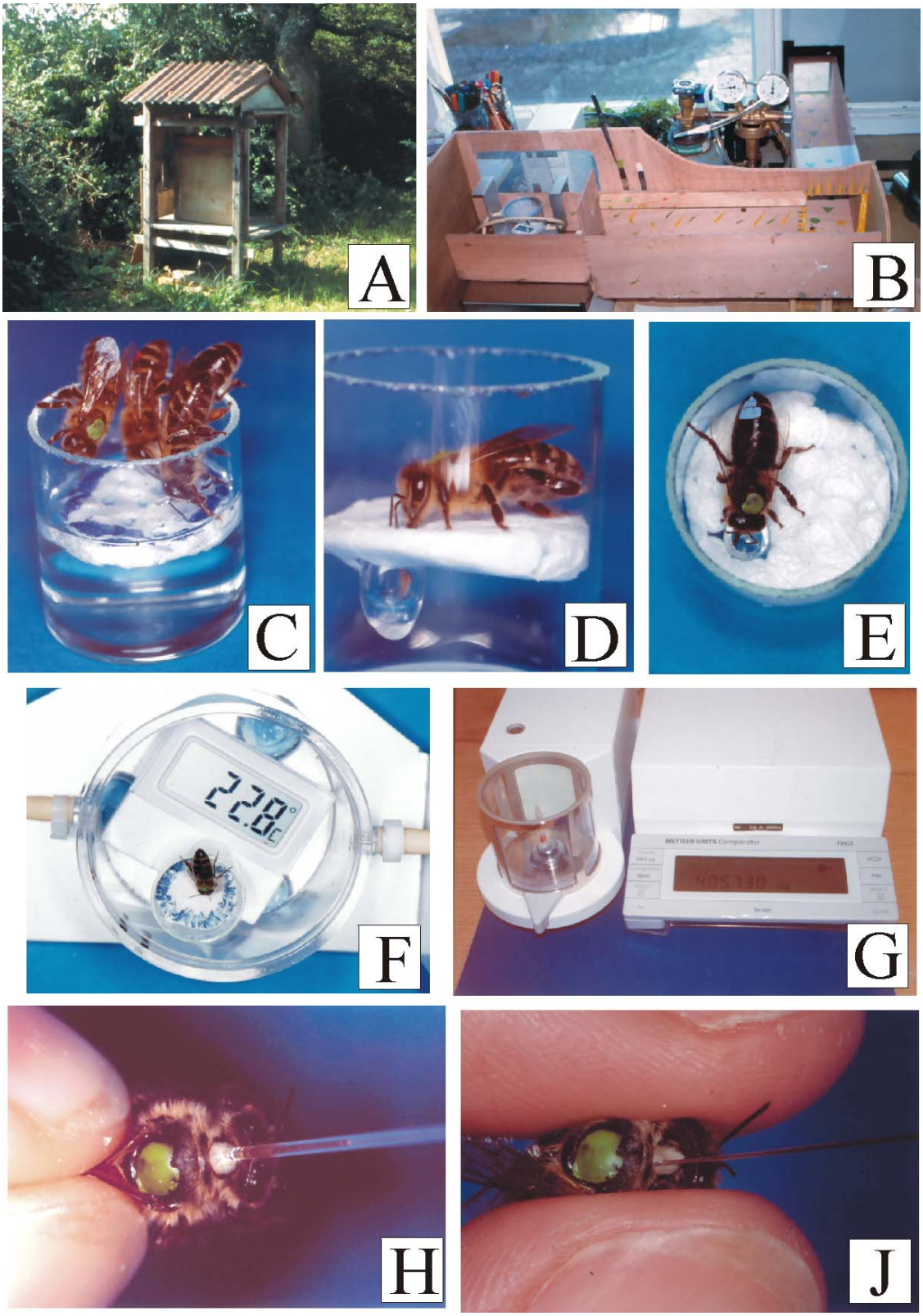

Fig. 4. Photographs of the observation hive (A), the tunnel, which the bees had to enter to reach the feeding place (B), bees foraging at the feedeer during the training stage (C), a bee sucking sugar solution during the experiment (D), a bee at the feeder during the experiment (E), a bee still collecting sucrose solution after it has been placed into the gas exchange chamber (F), the microbalance with a vial containing a pre-weight filter paper (G), haemolymph sampling with a microcap (H) and an injection into the neck membrane (J).

Read not to contradict and confute,
nor to believe and take for granted,
nor to find talk and discourse but to
weigh and consider.

Francis Bacon, "Essays"

3. RESEARCH

3.1. Haemolymph sugar titers: dependence on metabolic rate and *in vivo* measurement of maximal rates of trehalose synthesis

3.1.1. Introduction

During foraging, honeybees are able to increase their rate of oxygen consumption by more than 70-fold between rest and flight (Kammer and Heinrich, 1978). As the rapid processing of visual and other sensory information needs to be supported metabolically, their metabolism must be extremely flexible to balance ATP synthesis and degradation to co-ordinate the catabolic pathways (Wegener, 1996; Candy et al., 1997).

Honeybees use almost exclusively sugars as substrates for flight (Sacktor, 1970; Rothe and Nachtigall, 1989) and their brain is also highly specialised for carbohydrate oxidation (Tsacopoulos, 1995). As there appears to be no active transport of substrates from the midgut to the haemolymph (Crailsheim, 1988c), titers of substrates in the haemolymph must be kept sufficiently high to provide an adequate fuel supply. Indeed, total haemolymph sugar titers in the honeybee are among the highest recorded for any insect species (Fell, 1990).

Because honeybees store only limited amounts of glycogen in the flight muscle (Neukirch, 1982; Panzenböck and Crailsheim, 1997) and fat body (John, 1958; Panzenböck and Crailsheim, 1997), and use negligible quantities of amino acids as fuel (Micheu *et al*., 2000), they are almost exclusively dependent on intestinal and haemolymph energy supplies for most activities.

Previous investigations of haemolymph sugar titers in honeybees have been carried out under diverse and variable experimental conditions: assays were carried out with winter bees and summer bees, on bees ranging from previously starved to previously fed, from newly emerged to foragers and from active to immobilised. Consequently, haemolymph trehalose titers reported in the literature vary from 2 mg/ml (Bounias and Morgan, 1984) to 40 mg/ml (Bozic and Woodring, 1997), and glucose and fructose titers vary from 2 mg/ml (Abou-Seif *et*

al., 1993) to approximately 15 mg/ml (Fell, 1990; Leta *et al*., 1996). Haemolymph sugar titers in bees have been reported to change in response to the concentration of sugar solution imbibed (Crailsheim, 1988b; Abou-Seif *et al*., 1993), season (Crailsheim, 1988b) and behavioural pattern (Bozic and Woodring, 1997). On the basis of this high recorded variability, it has been suggested that there is no haemolymph sugar homeostasis in insects (Candy *et al*., 1997), even though regulating hormones have been found in many insect species (Gäde, 1996).

For honeybees, a possible explanation of the recorded variability in haemolymph sugar titers is that the different experimental conditions cause different titers of activity, resulting in metabolic differences. Such metabolic differences could have an important impact on proventriculus activity and thus on crop-emptying rate (Roces and Blatt, 1999). Depending on both the sugar concentration of the fed solution and the maximal rate of solution flow through the proventriculus (Núñez, 1969; Roces and Blatt, 1999), there may be an insufficient sugar supply for bees displaying high metabolic rates. Thus, the sugar transport rate through the proventriculus (the energy input) must be compared with the bee's metabolic rate (the energy output) to obtain a comprehensive view of the factors determining haemolymph sugar titers.

Purpose of the present study was to investigate the dependence of haemolymph sugar titers on metabolic rate and whether haemolymph sugar titers are regulated. Foraging bees were trained to collect controlled amounts of sucrose solution of different concentrations. After feeding, metabolic rate, crop-emptying rate and haemolymph sugar titers were recorded. Bees exhibiting a wide range of metabolic rates were compared to investigate whether differences in haemolymph sugar titers are caused by limits in the supply of sugar from the crop or in the rate of trehalose synthesis in the fat body.

3.1.2. Results

Metabolic rate showed a negative linear relationship with ambient temperature for bees walking inside in the respirometric chamber after feeding (Fig. 5). At low temperatures, metabolic rates were nearly as high as those recorded from flying honeybees (from 7 to 13 mlCO_2/h; Nachtigall *et al*., 1995; Balderrama et al., 1992). Metabolic rates ranging from 0.5 to 9.5 mlCO_2/h were recorded in the present study.

In order to compare the crop-emptying rate with the metabolic expenditure, the amount of sugar passing through the proventriculus in a given period was compared with that required to support the bee's metabolic expenditure over the same period. Metabolic expenditure was calculated directly from CO_2 production, since both flight and walking in bees is fuelled by

carbohydrate catabolism (respiratory quotient, RQ = 1; Rothe and Nachtigall, 1989), and given that 1 l of CO_2 is produced by the catabolism of 1.23 g of carbohydrate (Eckert, 1993).

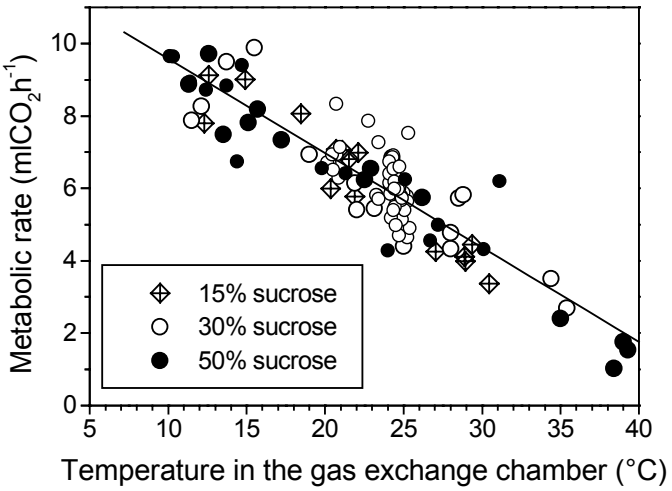

Fig. 5. Metabolic rate V_{CO2} of honeybees *versus* temperature T of the respirometric chamber, $V_{CO2} = 12.19 - 0.261*T$; $N = 110$, $r = -0.91$, $P < 0.0001$). Each point represents a single bee fed either 15%, 30% or 50% sucrose solution.

Fig. 6 shows the sugar transport rate of bees fed 30 µl of either 15%, 30% or 50% sucrose solution. The 'normlines' plotted indicate the expected relationship if the bees meet all their metabolic demands using sugar passed through the proventriculus. For bees fed 15% sucrose solution (Fig. 6A) sugar transport rates mostly lay above the normline, i.e. the foragers passed slightly more sucrose through the proventriculus than was needed to meet metabolic demands (comparison of linear regressions for measurements and normline: slopes were significantly different, ANCOVA: $F_{(1,36)} = 16.95$, $p < 0.001$, so that significance of intercept differences cannot be tested). Those bees with the highest metabolic rates (8 ml CO_2h^{-1} and above), however, showed sugar transport rates similar or even lower than predicted. However, note that the maximal fluid transport rate of the proventriculus in honeybees is 48 μlh^{-1} (Roces and Blatt, 1999), therefore the maximal amount of sugar that can pass through the proventriculus in bees fed 15% sucrose solution is 7.63 mgh^{-1}, which could not support metabolic rates higher than 6.2 $mlCO_2/h$.

A similar pattern was observed for bees fed 30% and 50% sucrose solution (Fig. 6B,C). However, while bees fed 30% passed significantly more sucrose through the proventriculus than needed to meet metabolic demands (comparison of linear regressions for measurements and normline: slopes did not differ statistically (ANCOVA, $F_{(1,58)} = 2.23$, $p > 0.1$), but the intercepts were statistically different (ANCOVA, $F_{(1,59)} = 23.19$, $p < 0.0001$), those fed 50% passed only as much as needed (slopes and intercepts were statistically similar; ANCOVA: $F_{(1,38)} = 0.023$, $p > 0.4$ and ANCOVA: $F_{(1,39)} = 1.22$, $p > 0.2$, respectively). Compared with bees fed 15% sucrose solution, the sugar transport rate through the proventriculus of bees fed

30% or 50% sucrose solution is sufficient to meet the highest metabolic rates. To support a metabolic rate of 10 mlCO_2/h, the proventriculus must transport 12.3 mgh^{-1} sugar or 36.38 µlh^{-1} of 30% sucrose solution, which is below the maximal recorded transport rate of 48 µlh^{-1} (Roces and Blatt, 1999).

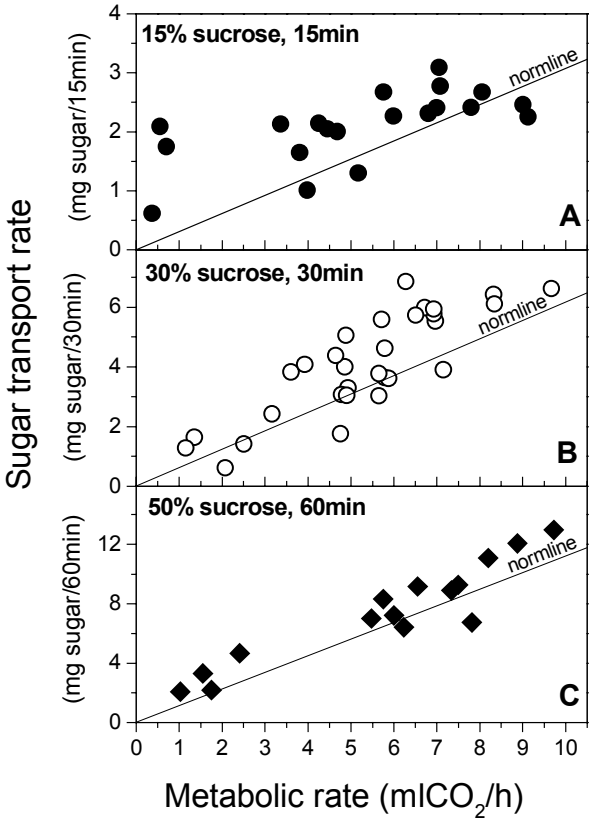

Fig. 6. Relationship between the sugar transport rate through the proventriculus and the CO_2 production of bees fed 30µl of one of three different sucrose solutions. Each point represents a single bee. The lines in the graphs are not regression lines but are lines representing the expected sugar transport rate, calculated from the measured CO_2 production, assuming all the metabolic expenditure to be met from the imbibed sucrose solution. (A) Bees fed 15% sucrose solution that remained in the gas exchange chamber for 15min. (B) Bees fed 30% sucrose solution that remained in the gas exchange chamber for 30min. (C) Bees fed 50% sucrose solution, measured over 60min.

Haemolymph sugar titers of the individuals plotted in Fig. 6A are presented in Fig. 7. Bees fed 15% sucrose solution showed a constant trehalose concentration of approximately 30mg/ml for metabolic rates ranging from 1 to 4.5ml CO_2h^{-1} (regression analysis: C_{Tre}= 30.75 – 0.36*V_{CO2}, N= 9, r= -0.129, P > 0.5). At higher metabolic rates, the trehalose concentration was observed to significantly decrease, reaching 5 mg/ml for bees with metabolic rates of 9 mlCO_2/h (regression analysis: C_{Tretot}= 37.02 – 3.34*V_{CO2}, N= 19, r= -0.822, P < 0.001). In contrast there was no relationship observed between metabolic rate and haemolymph sugar titers for glucose (mean ± SD = 7.9 ± 2.5 mg/ml; regression analysis: C_{Glutot}= 7.77 + 0.03*V_{CO2}, N= 19, r= 0.033, P > 0.5) or fructose (mean ± SD = 11.4 ± 2.7 mg/ml; regression analysis: C_{Frutot}= 10.98 + 0.08*V_{CO2}, N= 19, r= 0.082, P > 0.5). These patterns led to a significant decrease in total haemolymph sugar titers for metabolic rates higher than 4.5 ml CO_2h^{-1} (C_{Tot} =55.95 – 3.24*V_{CO2}; N= 19, r= -0.79, P < 0.0001).

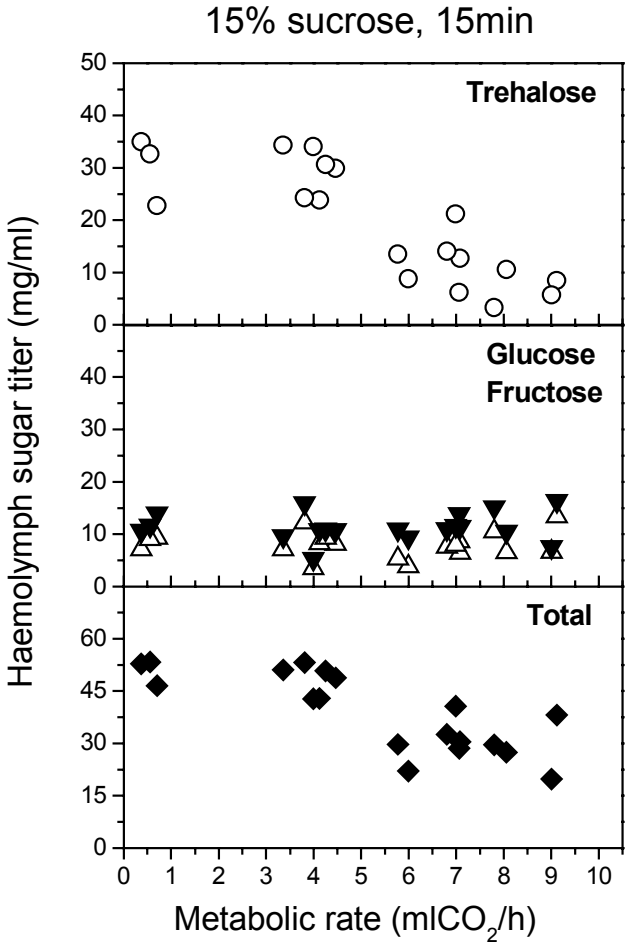

Fig. 7. Haemolymph sugar titers *versus* metabolic rate for bees fed 15% sucrose solution (same individuals as in Fig. 6A). Haemolymph samples were taken 15 min after feeding ended. Each symbol represents one bee. Trehalose (○), glucose (Δ), fructose (▼) and total (♦) sugar titers are plotted. Note the different scales on the ordinates. Total haemolymph sugar titers (C_{Tot}) decrease significantly with increasing metabolic rate V_{CO2} above 4.5 ml CO_2h^{-1} (see text).

Bees fed 30% sucrose solution (Fig. 8) also showed a constant trehalose concentration for metabolic rates up to 4.5 ml CO_2h^{-1} (regression analysis: C_{Tre}= 35.43 + 0.39*V_{CO2}, N= 7, r= 0.168, P > 0.5). Beyond this point trehalose concentration decreased with increasing metabolic rate (regression analysis: C_{Tretot}= 42.42 - 2.91*V_{CO2}, N= 33, r= -0.78, P < 0.001; Fig. 8). Glucose and fructose titers were constant at approximately 6 mg/ml for metabolic rates up to 4.5 mlCO_2/h (regression analysis: C_{Glu}= 6.01 + 0.07*V_{CO2}, N= 7, r= 0.048, P > 0.5; C_{Fru}= 3.14 + 1.37*V_{CO2}, N= 7, r= 0.606, P > 0.1), above which titers of both monosaccharides increased, reaching 15 mg/ml at the highest metabolic rates (regression analysis: C_{Glutot}= 3.35 + 0.92*V_{CO2}, N= 33, r= 0.553, P < 0.001; C_{Frutot}= 2.25 + 1.66*V_{CO2}, N= 33, r= 0.748, P < 0.001). The opposite patterns exhibited by titers of trehalose and the monosaccharides gave a constant total haemolymph sugar concentration, independent of metabolic rate (C_{Tot}= 48.01 - 0.32*V_{CO2}, N= 33, r= -0.110, P > 0.5).

For bees fed 50% sucrose solution (Fig. 9), the trehalose titers remained stable for the lowest metabolic rates (regression analysis: C_{Tre}= 44.77 - 1.18*V_{CO2}, N= 5, r= -0.445, P > 0.4). As for bees fed the lower concentrations of sucrose, trehalose titers then decreased with meta-

bolic rate, from the mean value of 43.5 mg/ml to approximately 15 mg/ml (regression analysis: C_{Tretot}= 47.55 – 3.23*V_{CO2}, N= 15, r= -0.848, P < 0.001). Glucose (6.6 ± 1.1 mg/ml) and fructose titers (5.4 ± 0.6 mg/ml) were initially relatively constant for metabolic rates up to 3.5 mlCO_2/h, increasing up to 17 mg/ml at the highest metabolic rates (C_{Glutot}= 4.38 + 1.09*V_{CO2}, N= 15, r= 0.721, P < 0.005; C_{Frutot}= 2.29 + 1.58*V_{CO2}, N= 15, r= 0.946, P < 0.001). The opposite patterns shown by titers of trehalose and the monosaccharides gave a constant total haemolymph sugar concentration independent of metabolic rate (C_{Tot}= 57.63 - 0.86*V_{CO2}, N= 15, r= -0.291, P > 0.2). Unfortunately, no measurements were performed between 3.5 and 5 mlCO_2/h for the 50% sucrose group.

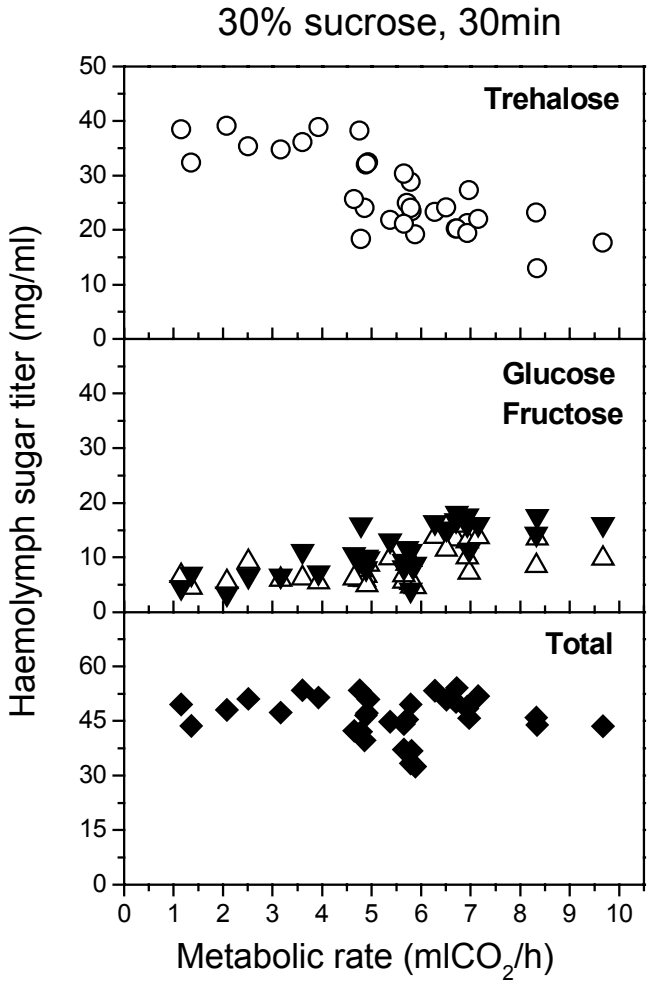

Fig. 8. Haemolymph sugar titers *versus* metabolic rate for bees fed 30% sucrose solution (same individuals as in Fig. 6B). Haemolymph samples were taken 30 min after feeding ended. Each symbol represents one bee. Trehalose (○), glucose (Δ), fructose (▼) and total (♦) sugar titers are plotted. Note the different scales on the ordinates.

Figs. 7 – 9 show, that independent of the concentration of the sucrose solution, the trehalose titers were stable at low metabolic rates (1 to 4.5 mlCO_2/h). At these metabolic rates mean trehalose titers were 29.7 ± 4.9 mg/ml for bees fed 15%, 36.4 ± 2.5 mg/ml for bees fed 30%, and 43.4 ± 2.3 mg/ml for bees fed 50% sucrose solution. The differences between the

groups are statistically significant. (ANOVA: $F_{(2,18)} = 19.42$; after Newman-Keuls comparisons, $P < 0.01$).

As indicated above, trehalose titers decreased for metabolic rates greater than 4.5 $mlCO_2/h$, and, with the exception of bees fed 15% sucrose, glucose and fructose concentrations increased. As the supply of sugar from the crop was not limited (see Fig. 6), it indicates that the rate of conversion of glucose to trehalose in the fat body was not high enough to maintain constant haemolymph trehalose titers at metabolic rates higher than 4.5 $mlCO_2/h$. The upper limit to the rate of trehalose synthesis therefore occurs at 5.54 mg glucose/h, i.e. 92.4 µg glucose/min.

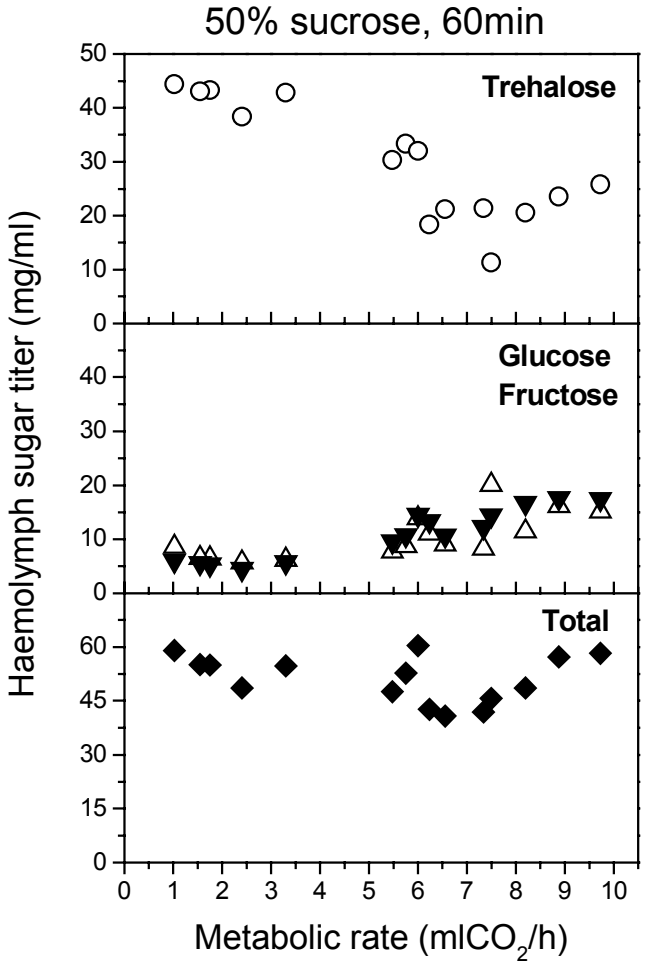

Fig. 9. Haemolymph sugar titers *versus* metabolic rate for bees fed 50% sucrose solution (same individuals as in Fig. 6C). Haemolymph samples were taken 60 min after feeding ended. Each symbol represents one bee. Trehalose (○), glucose (Δ), fructose (▼) and total (♦) sugar titers are plotted. Note the different scales on the ordinates.

3.1.3. Discussion

Dependence of sugar transport rates on metabolic rates

A linear dependence between sugar transport rate and metabolic rate of the foragers was detected in all investigated groups. The amount of sugar leaving the crop was similar or

slightly higher than that required to meet the bees' energy demands. Sugars leaving the crop reach the haemolymph *via* the midgut; the transport rate from midgut to haemolymph is very fast (Crailsheim, 1988a). Increasing consumption of haemolymph sugars with increasing metabolic rates was met by increasing sugar transport rates through the proventriculus, leaving the total haemolymph sugar titers unchanged. Bees fed 15% sucrose could not sustain metabolic rates higher than 6.2 mlCO_2/h in this way, because the maximal transport rate through the proventriculus is 48 µlh^{-1} (Roces and Blatt, 1999), which would not allow the supply of enough sugar at higher metabolic rates. Therefore, these bees must use haemolymph trehalose to meet their energy demands, thus leading to the observed decrease in trehalose and therefore in total haemolymph sugar titers. The highest flow of sugars through the proventriculus is in addition expected to prevent an increase in the monosaccharide titers at higher metabolic rates, as observed in our experiments, because not enough sugars can be passed to support the bee's metabolic expenditure.

Dependence of haemolymph sugar titers on metabolic rates

For bees fed 30 and 50% sucrose solution, the crop can deliver sufficient sugars to support the highest metabolic demands of the bees. Both glucose and fructose titers increased with increasing metabolic rates (see results), indicating that there was no delay in the movement of sugars from the midgut to the haemolymph. The most probable explanation for the decrease in trehalose titers at the highest metabolic rates (above 4.5ml CO_2h^{-1}) therefore is a limitation of the rate of trehalose synthesis in the fat body: a maximum value of 5.54 mg glucose/h was calculated. Núñez *et al*. (1974) showed that bees fed 50% sucrose solution had lower haemolymph trehalose titers and higher glucose and fructose concentrations than bees fed 7.5% sucrose. Although they did not measure metabolic rates, they suggested a limit to the rate of trehalose synthesis in the fat bodies of bees fed 50% sucrose solution. More recently, Woodring *et al*. (1994) suggested that haemolymph trehalose titers decreased in maximally active bees, because the fat body was unable to synthesise new trehalose as quickly as it was consumed, but no data were presented to allow comparison with the present results.

Decreasing trehalose titers and a concomitant increase in concentration of glucose and fructose was also found by Abou-Seif *et al*. (1993), working with caged bees assayed after 2h starvation periods. Unfortunately, detailed descriptions of the experimental treatment of the bees were lacking. However, it is noteworthy that haemolymph sugar titers at the beginning of their experiment were in the same range as those of our bees exhibiting low metabolic rates.

Fell (1990) noted that mean haemolymph sugar titers in the literature were generally similar, but that they showed high individual variability. Given the present results, it is likely that these are due to metabolic differences. In experiments on summer bees, Fell (1990) reported haemolymph concentrations of 20 mg/ml for trehalose, 16 mg/ml for glucose and 11 mg/ml for fructose, values which suggest that the samples were taken from bees with rather high metabolic rates.

Bozic and Woodring (1997) reported lower trehalose titers, but higher glucose and fructose concentrations in dancing than in resting bees. As it is very likely that dancing bees have higher metabolic rates than resting bees, these results correspond well with the present findings; their haemolymph sugar concentrations were in the same range as in the present study.

The present results show clearly that, when investigating haemolymph sugar titers of honeybees, and presumably of all other insects, measurement of the metabolic rate is important. If a comparison between two groups is made, one should take into account the effects of different handling protocols on the metabolism of the animals. For honeybees it is known that metabolic rate is dependent on temperature (Crailsheim *et al*., 1999), and that it increases with increasing crop load (Wolf *et al*., 1989). In addition, it has been suggested that a motivational drive controls the metabolic expenditure of foraging bees and modulates the effects of the carried load leading to changes in metabolic expenditure as a function of the reward flow rate, independent of the crop load (Núñez and Giurfa, 1996; Moffatt, 2000; Balderrama *et al*., 1992).

Dependence of haemolymph sugar titers on the sucrose solution concentration

Trehalose titers remained constant over low metabolic rates within each feeding group, however, this mean titer increased significantly with the sucrose solution concentration between groups. As the bees appear to adjust sugar transport rates through the proventriculus to equal metabolic rate, this phenomenon can not be explained by assuming that more sugars per unit solution enter the crop at higher sucrose solution concentrations. One possible explanation would be that the concentration of the sucrose solution affects the motivation of the foragers, and that this in turn influences the set point of the controlling system regulating trehalose titers. These results correspond well with those of Crailsheim (1988c) and Abou-Seif *et al*. (1993) who found for caged honeybees that individuals fed higher concentration sucrose solutions showed higher haemolymph sugar titers. Leta *et al*. (1996) stressed that there was no correlation between the amount of sugar solution carried in the crop and haemolymph sugar

concentration in individual bees, as they found constant titers of trehalose, glucose and fructose in colonies preparing to swarm and in those that were not.

Regulation of haemolymph sugar titers

Due to their high haemolymph sugar titers in comparison to vertebrates, it is still widely believed that there is no need for haemolymph sugar homeostasis in insects (Candy *et al*., 1997). However, regulatory hormones similar to those of vertebrates have been found in almost all insects investigated to date (Gäde *et al*., 1997; Van der Horst *et al*., 1999). In particular, honeybees with their large amount of food readily available in the hive or carried in the crop and their almost total lack of body reserves would not seem to need sugar-mobilising hormones. However, Woodring *et al*. (1993) found a factor in the corpora cardiaca (CC) of honeybees *Apis mellifera* that temporarily elevated total haemolymph sugars titers in *Periplaneta americana* and lipid concentrations in *Acheta domesticus*; but they could not find haemolymph reactions when injected in either fed or unfed honeybees. However, a significant increase in trehalose titers 30 and 60min after injection of CC extract, both in active bees moving about in glass vials and in immobilised bees, was found (Woodring *et al*., 1994). No change in glucose or fructose titers were observed. Both Maier *et al*. (1990) and Woodring *et al*. (1994) detected an increase in trehalose titers 30 min after glucagon injection; again no change in concentration of glucose and fructose was detected. Lorenz *et al*. (1999) showed that *Manduca sexta* adipokinetic hormone (Mas-AK) is present in the CC of the honeybee strain *Apis mellifera ligustica* but missing in *A. m. carnica*. Winter bees of both strains showed a weak trehalosemic response to injections of synthetic Mas-AKH after 60min, whereas summer bees of both races did not. Note, however, that experimental periods of 30 or 60min might be too long to observe a response due to the high metabolic rates of honeybees.

O´Connor and Baxter (1985) also detected an insulin-like material in the head of *A. mellifera*. However, Maier *et al*. (1988) noted that although the protein structure of this material is well explored, its biological role needs elucidation.

In the present study for bees fed 30 or 50% sucrose solution, total haemolymph sugar titers remained constant independent of metabolic rate, suggesting regulation of haemolymph sugar titers. Only in bees fed 15% sucrose solution did the total haemolymph sugar concentration decrease significantly (for metabolic rates higher than 4.5 $mlCO_2/h$). We also showed that trehalose concentration was stable for low metabolic rates (1 - 4.5 $mlCO_2/h$) in all three groups, but that the mean value depended on the concentration of the fed solution, suggesting that trehalose titers can be adjusted to the feeding conditions. To what extent hormones are

involved, and whether these observations are evidence of haemolymph sugar homeostasis or rheostasis, a state in which homeostatic regulation is present but there is a change in the regulated titer (Mrosovsky, 1990), can not be answered yet.

Regulation of haemolymph sugar titers could function as follows: with increasing metabolic rates the consumption of trehalose increases. This leads to a feedback signal to the proventriculus, which then releases more sucrose solution into the midgut. The cleavage of sucrose in the midgut allows both glucose and fructose to enter the haemolymph. Glucose is transformed to trehalose in the fat body, while the fructose is transformed into glucose in the haemolymph *via* a hexokinase and phosphoglucoisomerase (Candy *et al*., 1997). At low metabolic rates, bees are able to maintain constant trehalose titers, but at higher metabolic rates trehalose synthesis is not fast enough to balance the trehalose consumption. However, as trehalose titers decrease and titers of glucose and fructose increase at high metabolic rates, the total haemolymph sugar concentration remains constant providing the transport rate through the proventriculus is not limiting.

Further work needs to be carried out to elucidate the feedback loops involved in the regulation of haemolymph sugar titers in honeybees, but in such work careful standardisation of procedures for handling the bees is needed, and their metabolism should be monitored.

3.2. The control of the proventriculus: A dynamic process influenced by food quality and quantity?

3.2.1. Introduction

The control of feeding behaviour in insects is a complex process that involves the identification of food signals, ingestion, food processing and the regulation of meal frequency through negative feedback (Simpson et al., 1995). In almost all insects, the proventriculus regulates the transport rates of liquid and solid food between the crop and midgut, and since no absorption occurs in the crop (Maddrell and Gardiner, 1980), insects are only able to ingest food after it passed the proventriculus.

For non-social insects, it is expected that the feeding system is adapted to empty the crop into the midgut as fast as possible, in order to either metabolise the ingested food, or to transform it into body reserves. A number of factors that modulate crop emptying rates in non-social insects have been identified. In cockroaches, crop emptying rates were found to depend linearly on the concentration of the ingested sugar solution (Treherne, 1957); osmotic pressure in the crop lumen was indicated as the main regulatory factor (Davey and Treherne, 1963). Just as in cockroaches, feeding on more concentrated sugar solutions led to lower crop emptying rates in blowflies. Haemolymph osmotic pressure has been suggested as the controlling factor (Gelperin, 1966).

In contrast, the feeding system of social insects should be designed to retain as much food as possible in the crop, either for longer periods as in honeypot ants (Conway, 1977), or temporarily until the crop content is regurgitated in the nest, serving therefore as food for nestmates or brood (Cassill and Tschinkel, 1995; Seeley, 1995; von Frisch, 1927). Therefore it is expected that selective pressures led to a precise control of the proventriculus regulation in social insects.

In honeybees, the proventriculus activity need to be regulated to balance between supporting the single forager with sufficient energy and keeping as much of the collected nectar as possible for the colony. Body reserves are unlikely to be involved in the controlling mechanisms, because honeybees possess only negligible amounts (John, 1958; Micheu et al., 2000; Neukirch, 1982; Panzenböck and Crailsheim, 1997).

In honeybees, the passage of liquid food from the crop to the midgut was observed to depend on the concentration of the ingested sugar solution and on the locomotory activity of the individuals (Schreiner, 1952). Núñez et al. (1974) suggested haemolymph sugar titers, espe-

cially that of trehalose, as controlling variables for the proventriculus activity. More recently, Crailsheim (1988c) suggested an increase in haemolymph osmolarity as the factor controlling crop emptying rates in honeybees. The results of our previous study suggest the haemolymph sugar titers, rather than haemolymph osmolarity, as the factor controlling the activity of the proventriculus (Roces and Blatt, 1999).

As indicated above, various factors have been investigated in order to find those responsible for the regulation of the proventriculus activity. The factors can be categorised into output variables, input variables, and internal state variables (Fig. 10). In a previous paper we demonstrated that the crop emptying rates of foragers are linearly dependent on the output variable metabolism (Blatt and Roces, 2001a). Though we measured metabolic rates in our previous study concerning the regulation of the proventriculus (Roces and Blatt, 1999), for methodological reasons we have not used the same animals for determining crop emptying rates and metabolic rates. Therefore a basic problem of all previous investigations is that the metabolic rate of the investigated animals has never been controlled, especially as changing experimental conditions are very likely to influence the activity of the animals.

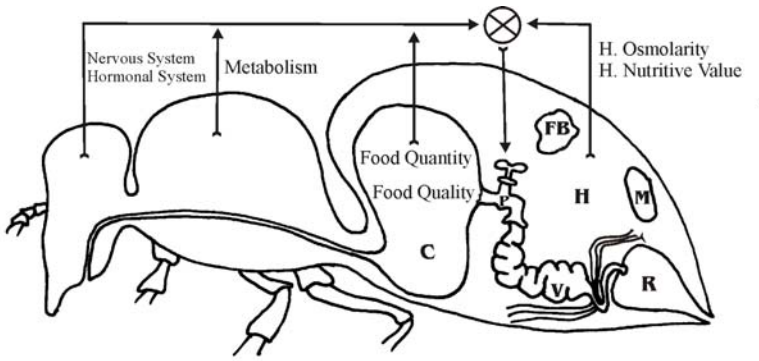

Fig. 10. Possible factors involved in the control of the proventriculus regulation. Differentiated according to input variables (food quality and food quantity), output variables (metabolism) and internal state variables (haemolymph osmolarity, haemolymph nutritive value). Body tissues are abbreviate as oesophagus (O), crop (C), midgut (V), proventriculus (P), fat body (FB), mitochondria (M), malphighian tubules (MP), rectum (R) and haemolymph (H).

The aim of the present investigation was to analyse the temporal dynamics of the proventriculus activity and to analyse to what extent the input variables food quality (concentration, molarity and viscosity of the fed solution) and food quantity have an impact on the regulation of the proventriculus. The next chapter (3.3) will focus on the effects of internal state variables (haemolymph osmolarity, haemolymph sugar titer and foraging motivation) on proventriculus activity. To achieve this, foragers were trained to collect defined amounts of different sugar solutions. Following feeding, they were dissected after fixed periods in order to measure crop emptying rates and haemolymph sugar titers. Between feeding and dissection the metabolic rate of every investigated forager was measured using open-flow respirometry. Therefore, we were able to directly measure crop emptying rate, metabolic rate and haemolymph

sugar titers for the same bee, and compare the effects of the different input variables on the activity of the proventriculus.

3.2.2. Results

Dynamic of the crop emptying rate

Fig. 11A presents an example of the dynamics of both crop emptying and rectal filling rates for bees collecting 30µl of 30% sucrose solution with a mean CO_2 production of 6 ml/h. As expected, the longer the time elapsed since feeding, the more fluid passes through the proventriculus, i.e. the lower the amount of solution which can be found in the crop. The time dependency of the production of rectal fluid corresponds well with that of the crop emptying rate, and thus provides a proper control for measurements of fluid quantity.

In order to compare the crop-emptying rate with the metabolic expenditure, the amount of sugar passing through the proventriculus in a given period was compared with that required to support the bee's metabolic expenditure over the same period. Metabolic expenditure was calculated directly from CO_2 production, since both flight and walking in bees is fuelled by carbohydrate catabolism (respiratory quotient, RQ = 1; (Rothe and Nachtigall, 1989)), and given that 1 l of CO_2 is produced by the catabolism of 1.23 g of carbohydrate (Eckert, 1993). The expected sugar transport rates, 'normlines', plotted indicate the expected relationship if the bees meet all their metabolic demands using sugar passed through the proventriculus.

In the first 30min (Fig. 11A), the fluid amount found in the crop was lower than expected based on the normline, thus indicating that the fluid flow through the proventriculus, and as a consequence the sugar transport rate, exceeded the metabolic requirements of the bees. After a period of 60min, the fluid flow through the proventriculus matched the metabolic consumption very precisely, so that the surplus of sugars ingested in the first 30min must have been levelled out in the following 30min.

According to the results of Fig. 11A, it would be expected that the surplus of sugars transported through the proventriculus in the first 30min, but not needed for the metabolism, should accumulate in the haemolymph, if the volume of the haemolymph had stayed constant (approximately 18µl (Crailsheim, 1985a)). If so, the haemolymph titer would have increased by c. 45mg sugar/ml. However, such an increase in the haemolymph sugar titers was not found (Fig. 11B). All three haemolymph sugar titers, trehalose (c. 22mg/ml), glucose and fructose (c. 11mg/ml) stayed constant for all periods of time and were not influenced by the overregulation (Fig. 11B, for statistics see figure legend).

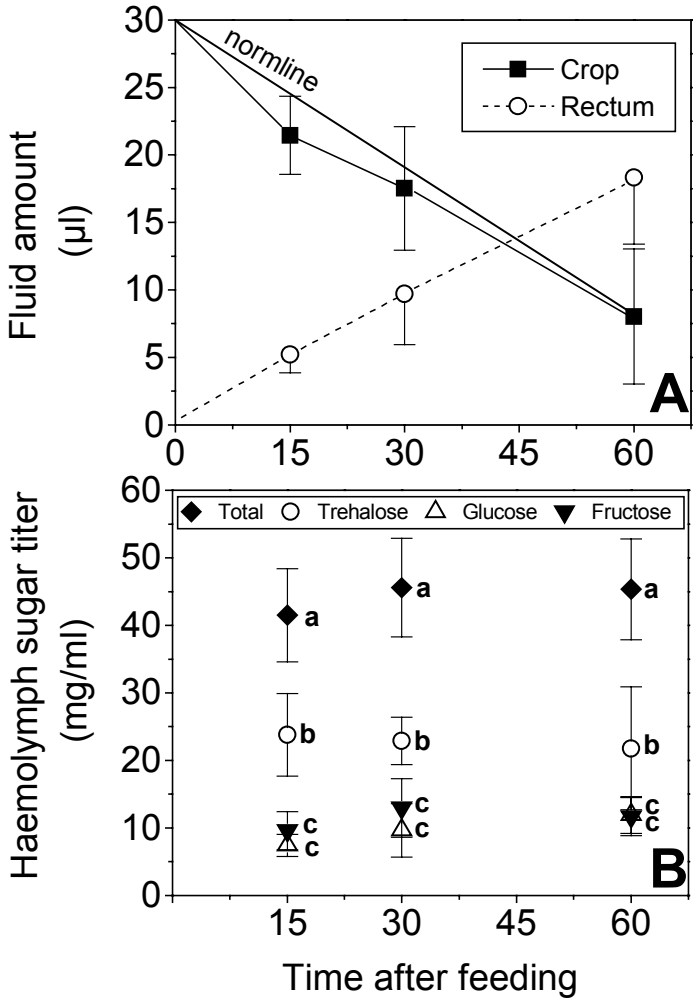

Fig. 11. Dynamic of both fluid amounts (A) and haemolymph sugar titers (B) of foragers collecting 30µl of 30% sucrose solution with mean metabolic rates of 6ml CO_2/h. Samples were taken 15, 30 or 60min after feeding ended. Given are means and SD for 10 to 15 bees per point.
Fig. 11A. The line in the graph is not a regression line but a "normline" representing the expected sugar transport rate, calculated from the measured CO_2 production (6mlCO_2/h), assuming all the metabolic expenditure to be met from the imbibed sucrose solution.
Fig. 11B. MANOVA, $F_{(6,164)}$ = 1.26, $p < 0.281$; symbols sharing the same letter are not statistically different (after Newman-Keuls comparisons, $\alpha = 0.05$).

In accordance with the findings for bees fed 30% sucrose solution, the sugar flow through the proventriculus for bees fed 50% sucrose solution also exceeded the metabolic requirements after 30min, and matched the metabolic requirements after 60min (data not shown). Bees fed 15% sucrose solution showed a similar but phase-shifted pattern: an overregulation after 15min and a precise regulation already after 30min (data not shown).

Dynamic as a function of the metabolic rate

To assess the accuracy of the regulatory system controlling the activity of the proventriculus, the relation between input (sugar transport rate) and output (metabolic rate) was analysed over time. Bees kept in the respirometric chamber for 15, 30 or 60min after feeding on 30µl of 30% sucrose solution were able to adjust their crop emptying rates to their metabolic rates over the wide range from 1 mlCO_2/h to 10 mlCO_2/h (Fig. 12, for statistics see figure legend). Therefore sugar amounts from less than 1.5 mg to more than 6mg were transported within 30min (Fig. 12B). However, a stable surplus of about 0.9 mg sugars left the crop after 15min

and 30min independently of the bees' metabolic rates. This amount of sugar surplus can support high metabolic rates (10 $mlCO_2/h$) for only 4½min but low metabolic rates (2 $mlCO_2/h$) for more than 20min.

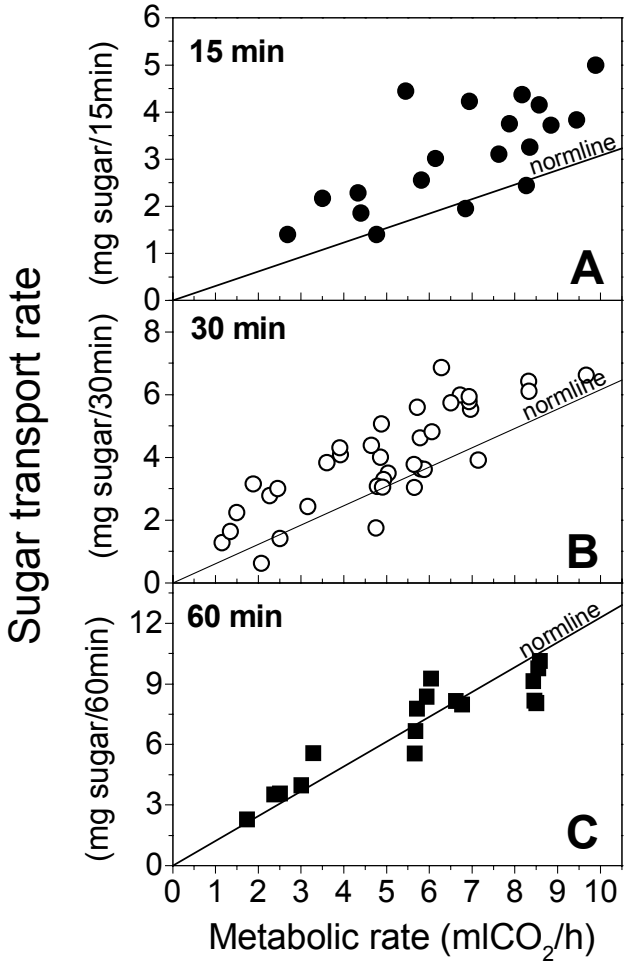

Fig. 12. Sugar transport rates in dependence on the CO_2 production of bees fed 30µl 30% sucrose solution for different times. Each dot represents a single bee. The lines in the graphs are not regression lines but are "normlines" representing the expected sugar transport rate, calculated from the measured CO_2 production, assuming all the metabolic expenditure to be met from the imbibed sucrose solution.
Fig. 12A. Bees remained in the gas exchange chamber for 15min. Linear regression: Y = 0.54 + 0.34 * X; n = 19, r = 0.73, p = 0.0004. By comparing the regression with the normline slopes were statistically equal (ANCOVA, $F_{(1,48)} = 1.57$, $p = 0.22$), but intercepts were statistically different (ANCOVA, $F_{(1,49)} = 54.54$, $p < 0.0001$).
Fig. 12B. Bees remained in the gas exchange chamber for 30min. Parts of the data was previously published (Blatt and Roces, 2001b). Linear regression: Y = 0.87 + 0.62 * X; n = 24, r = 0.83, p < 0.0001. By comparing the regression with the normline slopes were statistically equal (ANCOVA, $F_{(1,58)} = 3.25$, $p = 0.076$), but intercepts were statistically different (ANCOVA, $F_{(1,59)} = 32.07$, $p < 0.0001$).
Fig. 12C. Bees remained in the gas exchange chamber for 60min. Linear regression: Y = 1.58 + 0.93 * X; n = 17, r = 0.92, p < 0.0001.

After a period of 60min the sugar transport rates matched the metabolic consumption precisely.

Quality of the sugar solution fed: effects of sucrose concentration

After having shown that the sugar transport rate and thus the proventriculus regulation was strictly dependent on the output variable metabolic rate but that the accuracy of regulation was dependent upon time, we investigated whether the input variables food quality and food quantity influenced the proventriculus regulation directly by inducing changes in the bees' metabolic rate, or whether they had an independent effect.

To be able to compare the solution transport rates of the four fed concentrations, only bees with similar mean metabolic rates were investigated (Fig. 13, for statistics see figure legend). Since the amount of sugars dissolved in a given volume of solution increased with increasing

concentration the solution transport rate through the proventriculus decreased with increasing concentration of fed sucrose solution, while in 30min bees fed 7.5% sucrose solution passed about 25µl, bees fed 50% sucrose solution passed only about 8µl through the proventriculus.

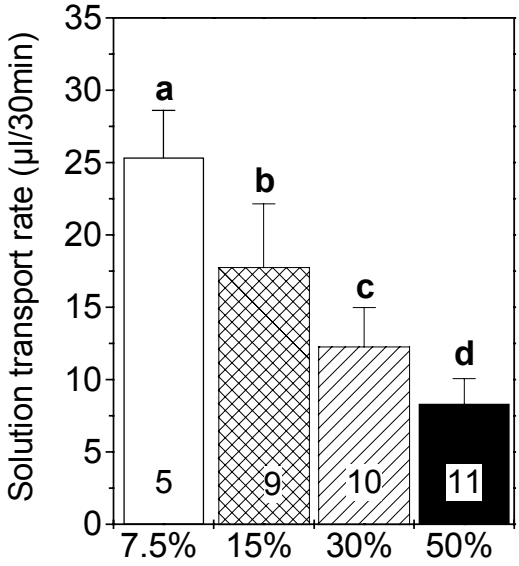

Fig. 13. Solution transport rates of bees collecting 30µl sucrose solutions of different concentrations (mean ± SD). The length of stay in the respirometric chamber was 30min. Mean metabolic rates ranged from 4.7 to 5.6 $mlCO_2/h$. Numbers in the columns represent the sample seize. MANOVA, $F_{(3,30)} = 34.13$, $p < 0.0001$; columns sharing the same letter are not statistically different (after Newman-Keuls comparison $\alpha = 0.05$).

The bees' mean metabolic rates increased with increasing concentration of the fed sugar solution, from $3.2mlCO_2/h$ for bees fed 7.5% solution over $4.9mlCO_2/h$ for bees fed 15% solution, and $5.7mlCO_2/h$ for bees fed 30% solution to $6.7mlCO_2/h$ for bees fed 50% solution. As the temperatures under which the bees were investigated were in the same range but not totally equal, this result can only be taken as a hint that bees fed higher concentrated sugar solutions have higher metabolic rates (Fig 14A).

Independently of the fed sucrose solution, the sugar transport rates were highly correlated with the metabolic rates (Fig. 14A). However, the sugar transport rates were not only dependent on the metabolic rates, but also influenced by the fed concentration itself. It has to be mentioned that for the groups fed 7.5% and 15%, the individuals with the highest metabolic rates were left out for statistical analysis (marked in Fig 14A with "*"). These bees finished the assays with empty crops, because amount and concentration of fed sugar solutions were not sufficient to support their rather high metabolic rates.

Though the slopes of the regression lines were equal to the normline for all groups, the regression lines of bees fed 30% or 50% sucrose solution had different intercepts from the normline, indicating that they constantly overregulate over the whole metabolic range (Fig 14A, for statistics see figure legend). In contrast, the regressions of bees fed 7.5% or 15% had equal intercepts to the normline, so that all three can be described as identical (Fig 14A, for statistics see figure legend).

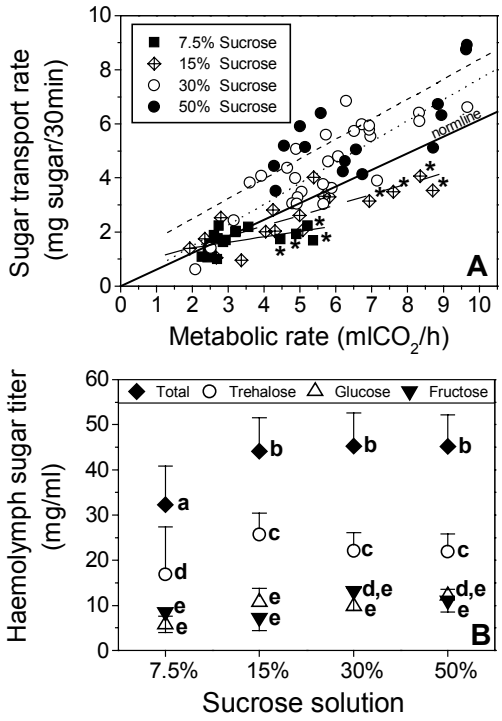

Fig. 14. Given are both, sugar transport rates in dependence on metabolic rates (**A**) and haemolymph sugar titers (**B**) of foragers collecting 30µl sucrose solutions of concentrations from 7.5% up to 50%. Bees remained in the gas exchange chamber for 30min.

Fig. 14A. The line in the graph is not a regression line but a "normline" representing the expected sugar transport rate, calculated from the measured CO_2 production, assuming all the metabolic expenditure to be met from the imbibed sucrose solution. Those individuals indicated with a star (*) were left out when comparing the groups and when determining the accuracy of regulation (Explanation, see results). Bees fed 7.5% or 15% had both statistically similar slopes (ANCOVA, $F_{(2,55)} = 1.38$, $p = 0.26$) and intercepts (ANCOVA, $F_{(2,57)} = 1.65$, $p = 0.20$) to the normline. Bees fed 30% or 50% had statistically similar slopes (ANCOVA, $F_{(2,71)} = 1.31$, $p = 0.28$) but different intercepts (ANCOVA, $F_{(2,73)} = 23.52$, $p < 0.0001$) to the normline. Mean metabolic rates increased with increasing concentration of fed sucrose solution (MANOVA, $F_{(3,76)} = 13.17$, $p < 0.0001$).

Fig. 14B. Bees which used up their crop content (*) and bees with metabolic rates above 7 $mlCO_2/h$ were exclude from the comparison of haemolymph sugar titers (mean ± SD) of the four groups (Explanation in the results). MANOVA, $F_{(9,172)} = 2.95$, $p = 0.0028$; symbols sharing the same letter are not statistically different (after Newman-Keuls comparisons, $\alpha = 0.05$).

The haemolymph sugar titers of the same individuals plotted in Fig. 14A are presented in Fig. 14B. Those bees indicated with "*" in Fig. 14A were left out when adding up the haemolymph sugar titers (explanation see above). Because it is known that the haemolymph sugar titers are dependent on the metabolic rates (Blatt and Roces, 2001a), only bees with metabolic rates up to 7 $mlCO_2/h$ were taken into account when calculating the mean. Bees fed sucrose solutions from 15% to 50% did not only have equal total haemolymph sugar titers (approximately 45mg/ml), but also equal titers of trehalose (c. 23mg/ml), glucose (c. 10mg/ml) and fructose (c. 10mg/ml) (Fig. 14B). Only bees collecting 7.5% sucrose solutions had a significantly lower total haemolymph sugar titer of about 32mg/ml, caused by a significantly lower trehalose titer of about 17mg/ml (Fig. 14B, for statistics see figure legend.).

As not only the nutritive value (amount of sugar per volume solution) but also both the viscosity and the molarity increase with increasing concentration of a sugar solution, the impact of molarity and viscosity on the sugar transport rate through the proventriculus was investigated separately.

Quality of the sugar solution fed: effects of molarity

As with increasing sugar concentration an increase in molarity is accompanied, in the following experiments the molarity of the feeding solutions were varied, while the nutritive val-

ues were kept constant. No statistical difference in the sugar transport rates have been observed for bees fed with approximately 1M sucrose or 1M trehalose to bees fed with approximately 2M glucose or 2M fructose (Fig. 15A, for statistics see figure legend). All solutions had the same nutritive value (30% sugar content), but were different in molarity due to being composed of either mono- or disaccharides. In comparison to the normline, all groups had, independently of the molarity of the fed sugar solution, statistically identical slopes, but different intercepts, indicating that the bees constantly overregulate over the whole range of metabolism (Fig. 15A, for statistics see legend).

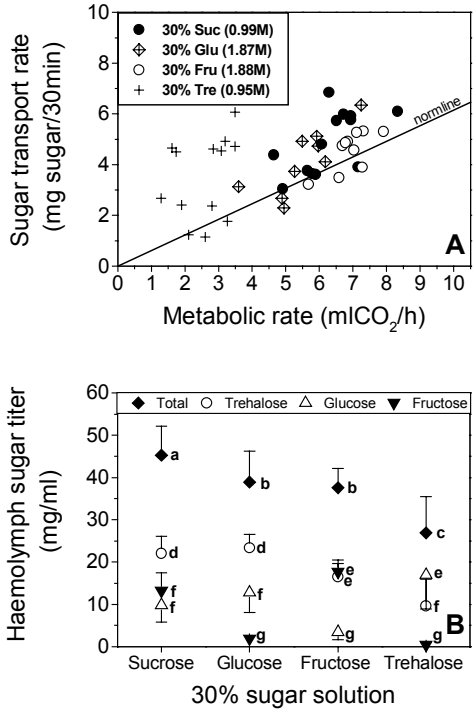

Fig. 15. Given are both, sugar transport rates in dependence on metabolic rates (**A**) and haemolymph sugar titers (**B**) of foragers collecting 30µl 30% solutions of sucrose (n = 15, mean metabolic rate = 6.2 $mlCO_2/h$), glucose (n = 12, mean metabolic rate = 5.3 $mlCO_2/h$), fructose (n = 9, mean metabolic rate = 6.8 $mlCO_2/h$) and trehalose (n = 11, mean metabolic rate = 2.8 $mlCO_2/h$). Bees remained in the gas exchange chamber for 30min.

Fig. 15A. The line in the graph is not a regression line but a "normline" representing the expected sugar transport rate, calculated from the measured CO_2 production, assuming all the metabolic expenditure to be met from the imbibed sucrose solution. By comparing the regression lines, the slopes and the intercepts of all groups were equal, so that the data could be described in terms of a single regression line (ANCOVA, $F_{(3,37)} = 0.16$, $p = 0.4$). However, when comparing the data with the normline, the intercepts differ (ANCOVA, $F_{(4,72)} = 12.52$, $p < 0.0001$), though the slopes were equal (ANCOVA, $F_{(4,68)} = 0.67$, $p = 0.38$).

Fig. 15B. Haemolymph sugar titers in dependence on the sugar solutions ingested by the bees (mean ± SD). MANOVA, $F_{(9,168)} = 19.95$, $p < 0.0001$; symbols sharing the same letter are not statistically different (after Newman-Keuls comparisons, $\alpha = 0.05$).

Although the sugar transport rates for bees fed trehalose were not statistically different from bees fed glucose, fructose or sucrose, bees fed trehalose constituted an exception. Under conditions otherwise the same, bees fed trehalose had comparably low metabolic rates and sugar transport rates with very high deviations. Some of these bees had sugar transport rates much higher than the metabolic rates would justify (Fig. 15A).

The haemolymph sugar titers of the same individuals plotted in Fig. 15A are presented in Fig. 15B. When interpreting these results it has to be considered that the glucose and fructose titers increase with increasing metabolic rate, while the trehalose titer decreases, leading to stable total haemolymph sugar titers (Blatt and Roces, 2001a). The total amount of haemolymph sugars was about 20% lower for bees fed monosaccharides (38mg/ml), in comparison to bees fed with sucrose solution (45mg/ml), even though the nutritive value per amount of

ingested solution was equal (Fig. 15B). Bees fed glucose solution had a similar titer of trehalose and glucose to bees fed with sucrose solution (22mg/ml), but only a very low titer of fructose (1.9mg/ml). Similar results to bees fed 30% solutions were found for bees fed either 50% sucrose solution or 50% glucose solution (data not shown). The haemolymph sugar titers differed, but the sugar transport rates were nevertheless equal.

In contrast to bees fed sucrose solution, bees fed fructose solution had both, a lower trehalose (16mg/ml) and a lower glucose titer (3.4mg/ml), but a very high titer of fructose (17.7mg/ml). However, the low trehalose titer and the very high fructose titer in bees fed fructose solution may be the result of the high mean metabolic rate (6.8 mlCO_2/h). In contrast bees fed glucose had a mean metabolic rate of only 5.3 mlCO_2/h.

Again, bees fed trehalose solutions were an exception. In spite of their low mean metabolic rate (2.8 mlCO_2/h), they possessed astonishingly low trehalose titers (9.6mg/ml), very high glucose titers (17mg/ml) and almost no fructose titer (0.3mg/ml) (Fig. 15B). It is worth mentioning that bees trained on trehalose arrive at the feeding place with haemolymph sugar compositions (25.5mg/ml trehalose, 10.9 mg/ml glucose and 8.1 mg/ml fructose) similar to those of sucrose fed bees (Fig. 15B).

Quality of the sugar solution fed: effects of viscosity

To be able to investigate for the effect of viscosity on the proventriculus activity independently from the sugar concentration fed, a 30% sucrose solution was thickened with Tylose to the viscosity of a 50% sucrose solution. Solution transport rates of bees fed 30% sucrose solution averaged 14.4 µl/30min, whereas those of bees fed 50% sucrose solution averaged only 10µl/30min (Fig. 16, for statistics see figure legend). Solution transport rates (14.7 µl/30min) of bees fed 30% sucrose solution, which was thickened to a viscosity of a 50% sucrose solution, were equal to bees fed a plain 30% sucrose solution.

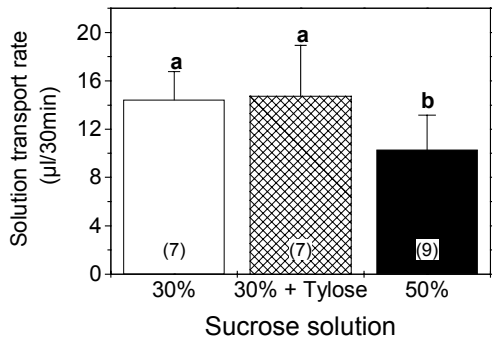

Fig. 16. Solution transport rates (mean ± SD) of bees collecting 30µl 30% sucrose solution added with tylose, in order to increase the fluid viscosity to equal that of a 50% sucrose solution are given (n = 7). Measurements of control bees collecting either 30% (n = 7) or 50% sucrose solution (n = 9) are also plotted. Mean metabolic rates of all groups averaged 8 mlCO_2/h. Bees remained 30min in the respirometric chamber. MANOVA, $F_{(2,20)}$ = 5.01, $p < 0.0173$; columns sharing the same letter are not statistically different (after Newman-Keuls comparisons, $\alpha < 0.05$).

This indicates that the sugar concentration rather than the viscosity influenced the crop emptying rate. For unknown reasons, bees fed the Tylose solution always had a metabolic rate above average.

Effects of the quantity of the sugar solution fed

No statistical difference in the sugar transport rates was observed for bees fed either 15, 30 or 50µl of 30% sucrose solution (Fig. 17, for statistics see figure legend), though the bees fed 50µl have a slight tendency towards higher sugar transport rates. Independently of the fed amount, bees showed the same overregulation, always observed after 30min.

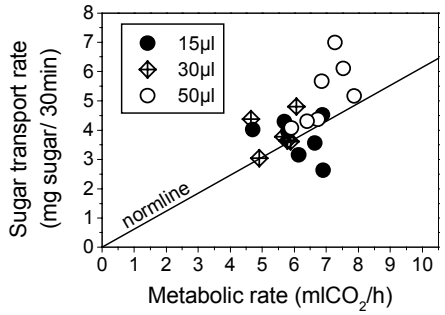

Fig. 17. Sugar transport rates of bees fed 15, 30 or 50µl 30% sucrose solution in dependence on the metabolic rate. Bees remained in the gas exchange chamber for 30min. The line in the graph is not a regression line but a "normline" representing the expected sugar transport rate, calculated from the measured CO_2 production, assuming all the metabolic expenditure to be met from the imbibed sucrose solution. The data could be described in form of a single regression; slopes: (ANCOVA, $F_{(2,14)} = 2.42$, $p = 0.13$); intercepts: (ANCOVA, $F_{(2,16)} = 3.22$, $p = 0.07$). The mean metabolic rate of bees fed 50µl was statistically higher than of bees fed 15, or 30µl (MANOVA, $F_{(2,17)} = 7.22$, $p = 0.0054$, after Newman-Keuls comparison $\alpha = 0.05$).

Bees fed 50µl reached significantly higher mean metabolic rates in comparison to bees fed 15µl or 30µl (Fig. 17, for statistics see figure legend). However, this result can only be taken as a hint that bees fed larger amounts of sugar solutions reach higher metabolic rates, as all bees were different individuals and the sample size was small.

3.2.3. Discussion

The proventriculus activity, a dynamic process dependent on the metabolism

Bee foragers adjusted their sugar transport rates to their metabolic rates under all investigated conditions. This is in good agreement with the findings of Crailsheim (1985a), who found that transport rates were always higher for running than for immobilised bees, and with the findings of Crailsheim (1988a), who found transport rates of 3.2µl for immobilised, 4.9µl for walking and 15.7µl for flying bees fed 2M glucose (nearly 30% sugar content).

However, the accuracy of adjustment of the sugar transport rate to the metabolic rate was not solely influenced by the metabolism of the animals, but also by the length of stay in the respirometric chamber. For non-social insects, Treherne (1957) and Venkatesh and Morrison (1980) found that crop emptying slowed down with time. In the honeybee Crailsheim (1988a),

Crailsheim (1988c) and Roces and Blatt (1999) reported higher crop emptying rates in the first 5min after feeding and steady ones from the minutes 5th to 60th after feeding. Therefore it seems very likely that the surplus of sugars found after 15 and 30min in the present investigation already left the crop in the first minutes after feeding, and was not levelled out until the 15th minute for bees fed 15% sucrose solution, and the 30th minute for bees fed 30% and 50% sucrose solution. (No data were taken after 5min in this study due to the difficulties of measuring metabolic rates in such short periods). While Crailsheim (1988a) and Crailsheim (1988c) did not offer any explanation for the extraordinarily high crop emptying rates in the first minutes after feeding, Roces and Blatt (1999) suggested a delay in the control system regulating crop emptying rates to be responsible for it. However, since intervals as long as 15 to 30min elapsed until the surplus of sugars was levelled out, a time delay in the control mechanism seems to be unlikely. Especially, as it is known that the way of the sugars from crop via midgut to haemolymph (Crailsheim, 1988b), from haemolymph to the fat bodies, and from the fat bodies back to the haemolymph is much quicker (Gmeinbauer and Crailsheim, 1993). An even more important argument against a time delay is that the bees are able to precisely adjust their sugar transport rates to their metabolic rates in the same space of time.

A conceivable hypothesis concerning the overregulation would be that bees engaged in regular foraging activity pass sugar solution through the proventriculus in advance (a feed-forward mechanism), because they need an extra amount for the energy-consuming take-off and further flight. A possible explanation for the more accurate regulation with time would be that the foraging motivation of the bees slowly decreases with time inside the chamber, thus reducing the need of a feed-forward mechanism.

Whereabouts of the ingested sugars

The haemolymph sugar titers stayed constant, independently of the length of stay in the respirometric chamber, and therefore independently of the accuracy of adjustment of the sugar transport rate to the metabolic rate. An increase in haemolymph volume could a priori be responsible for the observed constancy in haemolymph sugar titers, thus masking the absolute increase in haemolymph sugar content. This is, however, very unlikely. To level out the surplus of about 1mg sugars, the haemolymph volume would show a two-fold increase.

A total incorporation of the extra amount of sugars into body reserves would produce higher amounts of glycogen in the flight muscle (Neukirch, 1982), (Panzenböck and Crailsheim, 1997) or fat body (John, 1958; Panzenböck and Crailsheim, 1997), than have ever been measured.

As Gmeinbauer and Crailsheim (1993) found practically no loss of radioactivity by defecation, and we also never detected significant amounts of sugars in the rectum (unpublished results), the loss of the sugars via the rectum is also very unlikely.

Though it is known that the passive transport from midgut to haemolymph is rather quick and sufficient to supply the bee's metabolism (Crailsheim, 1988b), it is nevertheless possible that sugars temporarily accumulate in the midgut. And indeed has Crailsheim (1988a) found that the mean midgut weight from empty bees was 12mg, while that of fed bees averaged 15mg after a 15min-flight. However, until no measurements of the total sugar amount in the midguts are made, it remains unclear whether the sugar surplus will be found in the midgut.

Observable effects by changing the concentration of the fed sucrose solution

When discussing the effect of increased concentration of fed sucrose solution, it is important to have in mind that nutritive value, molarity, viscosity and "stimulating power" increase simultaneously with increasing sugar concentrations (Gelperin, 1966). In the following, rather than "stimulating power" the term "motivation" after McFarland (1989) should be used.

As in previous investigations, (Treherne, 1957; Davey and Treherne, 1963; Gelperin, 1966; Venkatesh and Morrison, 1980; Crailsheim, 1988a; Crailsheim, 1988c; Roces and Blatt, 1999), a decrease in the solution transport rate with increasing concentration of the fed sugar solution was found.

In the present investigation we were able to show that with increasing concentration of the fed sucrose solution, the metabolic rates of the bees increased, thus leading to higher sugar transport rates. Furthermore, the sugar transport rates increased with increasing concentration of the fed sucrose solution, independently of the metabolic rates.

In the honeybee, Crailsheim (1988c) found that the amount of transported sugars increased, while the transported volume decreased with increasing concentration. Crailsheim (1988a) also found that glucose transport from midgut to haemolymph was similar when the bees were fed 1 or 2M glucose, but was larger when the bees were fed 3M glucose.

In a previous study on honeybees, we found that independently of the concentration of the sucrose solution collected by the bees, there was a close match between sugar transport rate and the energy expenditure (Roces and Blatt, 1999). We did not detect an increase in sugar transport rate with increasing concentrations of the fed solution, probably because of the small amounts involved, and the high interindividual variability.

By taking the data of Gelperin (1966) and converting the crop emptying rates (volumes) into sugar transport rates, we found that the sugar transport rate was –with about 0.13mg/h –

almost equal for blowflies fed 1.5M or 2M fructose solution, but was –with 0.18mg/h –greater for blowflies fed 3M fructose. By proceeding in the same way with the data from Treherne (1957), we found that cockroaches passed about 1.25mg/h or 0.4mg/h sugar respectively through the proventriculus after feeding on either 2.77M or 0.11M glucose. From this results we can conclude that also for non-social insects, an increase in the sugar transport rate through the proventriculus with increasing concentration was found. In addition, the investigated animals have a much lower metabolism than honeybees, because of their much lower sugar transport rates. Whether the sugar transport rates observed in the studies of Crailsheim (1988a), Crailsheim (1988c), Gelperin (1966) and Treherne (1957) were really due to the increased concentration of the fed sugar solutions or to a concomitant increase in the metabolic rates, can not be answered.

Distinguishing between nutritive value and molarity of the fed sugar solution

In spite of the different molarity, bees collecting either 30% sucrose, 30% glucose or 30% fructose solutions showed equal sugar transport rates. As sucrose is a disaccharide and glucose and fructose are monosaccharides, the latter are almost twice as molar as sucrose. In good agreement we found identical responses for bees fed either 30% sucrose or 30% glucose in a previous study as well (Roces and Blatt, 1999). Likewise, Crailsheim (1985b) found that the solution transport rate in bees fed 2M sucrose solution was more or less equal to that of bees fed a 4M mixture of glucose and fructose solution. He concluded that sucrose was cleavaged in the crop and assumed that the molarity is the controlling factor in the regulation of the proventriculus activity. However, HPLC measurements of crop sugars showed that under our experimental conditions, nearly no cleavage of sucrose took place in the crop even 60min after feeding (unpublished results). This leads to the assumption that, rather than the molarity, the nutritive value of the fed solution influenced the proventriculus activity.

Crailsheim (1985b) found that a 50% replacement of glucose with non digestable inosit has no influence on the transport velocity and that bees fed a mixture of 1.5M mannitol and 0.5M glucose showed identical solution transport rates to bees fed 2M glucose solution (Crailsheim, 1988c). He therefore concluded that the nutritive value is not responsible for the transport rate from crop to midgut. However, he also reported very low haemolymph glucose titers for bees fed the mixture of 1.5M mannitol and 0.5M glucose in comparison to bees fed 2M glucose solution. Since a low haemolymph glucose titer correlates with a low metabolic rate (Blatt and Roces, 2001a), the differences found may be due to differences in the bee's metabolic demands. Particularly, as Crailsheim et al. (1999) himself reported that immobilised bees show

unexpected metabolic rates. In addition, we found that immobilised bees show a less precise adjustment of crop emptying rates to metabolic rates (unpublished results).

The low metabolic rates and highly variable crop emptying rates found in bees feeding on trehalose solution is accompanied by low total haemolymph sugar titers. The absence of fructose is not surprising, as trehalose is a disaccharide built from two glucose molecules, but high glucose and low trehalose titer are usually only found in very active bees (Blatt and Roces, 2001a). When bees are kept in a cage for 48h and feed on trehalose only, their midgut is extremely inflated and the animals hardly move at all. This indicates problems not only with the absorption of sugars, but also with fluid regulation.

Like sucrose, trehalose is split only to a very small extent in the crop, but in contrast to sucrose, which is almost totally split in the midgut, we found trehalose titers of about 70mg/ml in the midgut (unpublished results). In addition, we found very low trehalose titers in the haemolymph of bees feed a trehalose solution. This indicates that there must be both problems with the cleavage of trehalose in the midgut, and with the diffusion out of the midgut into the haemolymph. Following this assumption one would expect that in order to achieve an equilibrium between midgut and haemolymph, water streams into the midgut, thus leading to an increase in the midgut volume. It is astonishing that the bees forage under these conditions. As bees trained on trehalose arrive at the feeding place with haemolymph sugar titers similar to sucrose fed bees, we assume that they feed on honey in the hive and thus reduce the negative effects of trehalose feeding.

Distinguishing between nutritive value and viscosity of the fed sugar solution

A thickening of a 30% sucrose solution to the viscosity of a 50% sucrose solution had no influence on the sugar transport rate. In good agreement Crailsheim (1985a), who increased the viscosity's of 0.5 or 1M sucrose solutions to that of a 2M sucrose solution, found that the viscosity did not influence crop emptying rates. Not only in bees, but also in other insects investigated so far, the viscosity had no considerable effect on the crop emptying rate (Gelperin, 1966; Davey and Treherne, 1963). In contrast to these results, in a previous study we found unexpectedly higher crop emptying rates and shorter exhaustion times for bees fed with a 15% sucrose solution, the viscosity of which was increased to a 50% sucrose solution, in comparison to bees fed with a plain 15% sucrose solution (Roces and Blatt, 1999). Though we measured similar mean metabolic rates for both groups (Roces and Blatt, 1999), we now nevertheless suggest that the increased crop emptying rates were due to higher metabolic rates, because we used different individuals for the measurements of crop emptying and

metabolic rates (Roces and Blatt, 1999). This argument is supported by the present investigations, showing that the metabolic rates of bees fed Tylose solutions were found to be above average. This might be an indication that bees fed thickened solutions had motivations like bees fed higher concentrated food, but that this has no influence on the regulation of the proventriculus.

Food quantity

In the present investigation there were no statistical differences concerning the sugar transport rates between the three quantities fed. However, a slight tendency towards higher sugar transport rates with increasing quantity was observable. Crailsheim (1988c) as well found a weak correlation between passage rate and volume of fed solution. As we found in the present study that bees fed 50µl rather than 15µl or 30µl had higher metabolic rates and as Crailsheim (1988c) did not monitor metabolic rates, it can not be finally concluded if the quantity fed has an influence on the sugar transport rate. However, from the present results and from the results of Crailsheim (1988c), we can conclude that the effects of food quantity, if any, are at best minimal.

Haemolymph sugar titer and the regulation of the proventriculus activity

In a previous paper, we showed that independently of the fed sucrose solution, trehalose, glucose and fructose titers were observed to remain constant for metabolic rates from 0 to 4.5 mlCO_2/h. For higher metabolic rates, the trehalose titer decreased and the titers of glucose and fructose increased. This dynamic resulted from an upper limit in the capacity of the fat bodies to synthesise trehalose (Blatt and Roces, 2001a). The opposite courses of trehalose and the monosaccharides as a function of metabolic rate, which lead to stable total haemolymph sugar titers, have to be considered when a comparison of haemolymph sugar titers is aimed to, especially if the groups to be compared had different metabolic rates.

In the present investigation we showed that, as long as the bees do not exhaust their crop content, the haemolymph sugar titers were independent of the time after feeding and the concentration of the fed sucrose solution, with exception of bees fed 7.5% sucrose solution. The difference for bees fed 7.5% solution was mainly due to lower trehalose titers. When bees are fed low concentrated sugar solutions, they are limited by the maximal solution transport rate (Roces and Blatt, 1999).

In comparison to bees fed sucrose solutions, bees collecting glucose or fructose solutions had lower total haemolymph sugar titers. These differences were due to low glucose titers in bees fed fructose solution, and to low fructose titers in bees fed glucose solution. To under-

stand this pattern, it should be considered that sugar absorption is just dependent on the concentration gradient (Turunen, 1985), and that both glucose and fructose absorption rates are similar and independent of each other (Crailsheim, 1988b). As a consequence, both glucose and fructose (either fed as a mixture to the bees, or resulting from the cleavage of the fed sucrose) would show up in the haemolymph. If a monosaccharide solution is fed to the bees, only this monosaccharide would show up in the haemolymph. If fructose is fed, it will be converted into glucose (Maurizio, 1965), which in turn will be transformed into trehalose in the fat bodies. If glucose is fed, it will be directly converted to trehalose. Accordingly, Gmeinbauer and Crailsheim (1993) detected labelled trehalose after feeding bees with labelled glucose, but he found no incorporation of radioactivity into fructose. Finally, the comparably high fructose titer and low trehalose titer in bees fed fructose in our study is caused by their high metabolic rate.

Maurizio (1965) found that the haemolymph sugar composition of the honeybee is dependent on the diet. However, we showed that although the glucose and fructose titers varied in dependence on the fed sugar solution, thus leading to different total haemolymph sugar titers, the trehalose titer remained unaffected.

The trehalose titer was shown to be unaffected by the time after feeding, the accuracy of regulation, and the concentration of fed sugar solution. In addition, neither the solution transport rate from crop to midgut (Roces and Blatt, 1999), nor the capacity of the fat bodies to synthesise trehalose reached its upper limit under the experimental conditions (Blatt and Roces, 2001a). Taking all together, we suggest that the trehalose titer is regulated in the honeybee and it represents the controlled variable in the feedback loop responsible of the regulation of the proventriculus.

Summing up, we showed that the sugar transport rate through the proventriculus is mainly dependent on the metabolism. However, though the foragers are able to adjust their sugar transport rates to their metabolic rates, the match between both is not numerically exact. Over the whole range of metabolism, a fixed surplus of sugars leaves the crop. This fixed amount of sugars is, though independent from the metabolic rate, dependent on the nutritive value and quantity of the fed sugar solution and on the time after feeding. But it is independent of those factors that have no impact on feeding energetic, as the molarity and viscosity of the fed sugar solution. In the next chapter (3.3) we will present results from experimental manipulations of the variables involved in this regulatory system, in order to address the question to what extent an increase in haemolymph osmolarity or an increase in the haemolymph nutritive value, performed via injections, influence the proventriculus regulation.

3.3. The control of the proventriculus: Feedback mechanisms and functional questions

3.3.1. Introduction

In the previous chapter (3.2.) we focused on the temporal dynamics of the proventriculus activity and on the impact of the input variables food quality and food quantity on the regulation of the proventriculus. However, the precise mechanisms involved in the proventriculus regulation remain to be elucidated. There is good reason to suspect that the haemolymph may provide central information for the control of nutrient intake. Unlike other sources of feedback involved in the control of ingestion, the haemolymph provides a constantly updated indication of an insect's nutritional state (Simpson and Raubenheimer, 1993). For honeybees, while Crailsheim (1988b) suggested an increase in haemolymph osmolarity, Blatt and Roces (2001a), Blatt and Roces (2001b), Núñez et al. (1974) and Roces and Blatt (1999) suggested haemolymph sugar titers, especially that of trehalose, as controlling variables for the proventriculus activity. We showed that the trehalose titer remained unaffected by the kind of sugar used in the fed solutions, the time after feeding, the accuracy of regulation, and the concentration of the fed sugar solution (Blatt and Roces, 2001a; Blatt and Roces, 2001b). However, these are only indications that the trehalose titer might be involved in the control system regulating the activity of the proventriculus. In order to investigate whether the osmolarity or the nutritive value of the haemolymph is the factor controlling the proventriculus activity, both, Crailsheim (1988b) and also we, Roces and Blatt (1999), injected either metabolisable or non-metabolisable sugars into the haemolymph. While the results of Crailsheim (1988b) allow the assumption that the haemolymph osmolarity is the controlling variable in the proventriculus regulation, our results Roces and Blatt (1999) supported the assumption that the nutritive value of the haemolymph is the controlling variable. Neither of us, however, had monitored the metabolic demands of the investigated animals. As we now know that the proventriculus activity is strongly dependent on the bee's metabolic rates (Blatt and Roces, 2001a; Blatt and Roces, 2001b), the first aim of the present study was to manipulate the control system regulating the activity of the proventriculus via injections of metabolisable and non-metabolisable sugars, whereby the metabolic rates of all investigated bees should be measured.

With regards to the relationship between proventriculus activity and metabolic expenditure, we showed that although bee foragers were able to precisely adjust their sugar transport rates to their metabolic rates, the match between both was, however, not numerically identical

(Blatt and Roces, 2001a; Blatt and Roces, 2001b). Over the whole range of metabolism, a fixed surplus of sugars left the crop during the first minutes after feeding. This fixed amount of sugars was dependent on all input variables that positively influence feeding energetics (nutritive value and food quantity), but independent of those which have no influence on it (viscosity and molarity). To account for this complex pattern, we suggested the involvement of feed-forward mechanisms in the overregulation of the activity of the proventriculus. If so, foragers would release the extra amount of sugars in advance ”to prepare” for the imminent take-off and flight.

While the first part of the present paper deals with the mechanisms underlaying the control of the proventriculus, the second part addresses functional questions about the overregulation. In a first experiment, we investigated whether the bees release an extra amount of sugar solution only immediately before leaving for the hive. In a second experiment, groups of bees were trained to fly different distances to reach the food source, under the hypothesis that the distance to be covered may influence the surplus amount released prior take-off. In a third experiment, trained foragers were able to reach the food source without flight just by walking a short distance. This experiment allowed to control for potential effects of flight on the regulation of the proventriculus activity.

3.3.2. Results

Preliminary investigations

Fig. 18 presents examples of metabolic rate measurements for an uninjected bee (Fig. 18A), a bee successfully injected with 1µl Ringer after 15min (Fig. 18B), and a bee injected with 1µl Ringer after 15min, whose metabolic rate showed alterations and finally collapsed totally (Fig. 18C). While the uninjected bee and the bee successfully injected had mean metabolic rates of about 6.5 $mlCO_2/h$, the bee with collapsed metabolism had a mean metabolic rate of only 4.0 $mlCO_2/h$, though its metabolism before injection was similar to that of the other bees.

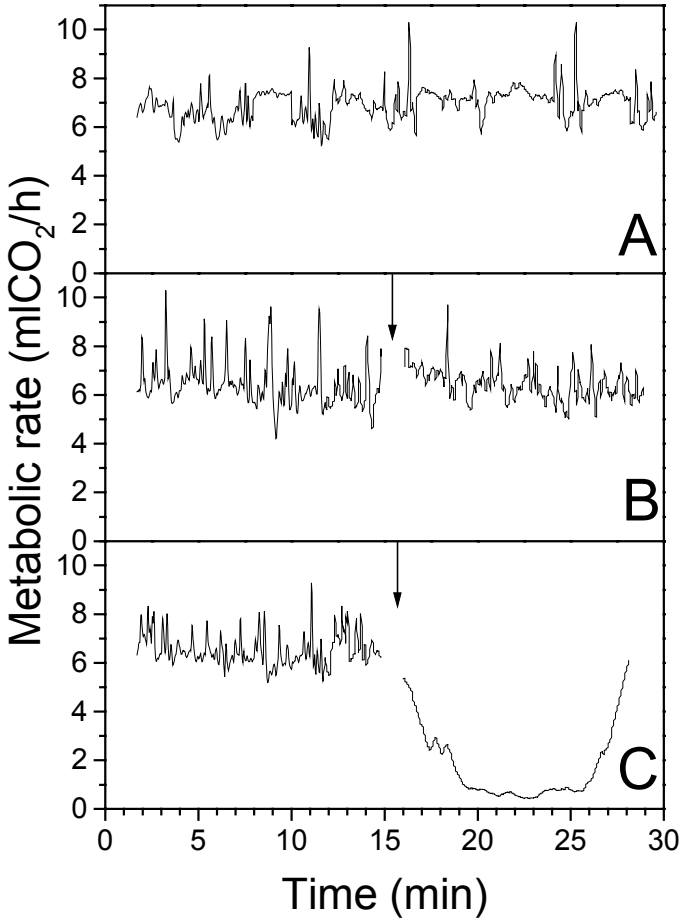

Fig. 18. Examples of 30min measurements of metabolic rates for bees fed 30µl 30% sucrose solution. Without further treatment (**A**); successfully injected with 1µl Ringer after 15min (**B**); with collapsing metabolism after the injection of 1µl Ringer after 15min (**C**). The arrows indicate when injections were administered; shortly before and after injection no metabolic rates could be measured.

Haemolymph sugar composition 15min after feeding depended on the region of sampling (Fig. 19A, for statistics see figure legend). While glucose and fructose titers were much higher in the gaster (c. 21mg/ml), than in the neck (c. 9mg/ml), the trehalose titer was similar in both compartments (c. 21mg/ml), consequently leading to a total haemolymph sugar titer of 40mg/ml for the neck sample and of 63mg/ml for the gaster sample.

Bees injected with 1M trehalose had higher haemolymph trehalose titers 3min after injection in comparison to control bees injected with Ringer (Fig. 19B, for statistics see figure legend), whereas the glucose and fructose titers showed no differences, thus serving as a direct proof that the injected solutions really reached the haemolymph. Although the trehalose titer increased by about 10mg/ml, it should have increased by double the amount (21mg/ml) if the total amount of injected solution had been present in the haemolymph. However, as the injected amount of sugars (0.38mg) was only sufficient to cover the observed metabolic demands for about 4 min, it is not surprising that some of the injected trehalose is not detectable any more after 3min.

Though haemolymph sugar compositions were different, regardless of whether haemolymph sugar samples were taken from the gaster or from the neck membrane, bees injected with 1µl 1M trehalose into the neck membrane or gaster showed equal crop contents 15min after injection (Fig. 19C, for statistics see figure legend). Bees injected either into the neck membrane or into the gaster had both mean crop contents of 20µl, whereas uninjected bees had a mean crop content of 16µl. Since the metabolic rates in bees belonging to all three groups were similar, the trehalose injection led to a similar decrease in the solution transport rate from crop to midgut independently of the compartment into which the injections were administered.

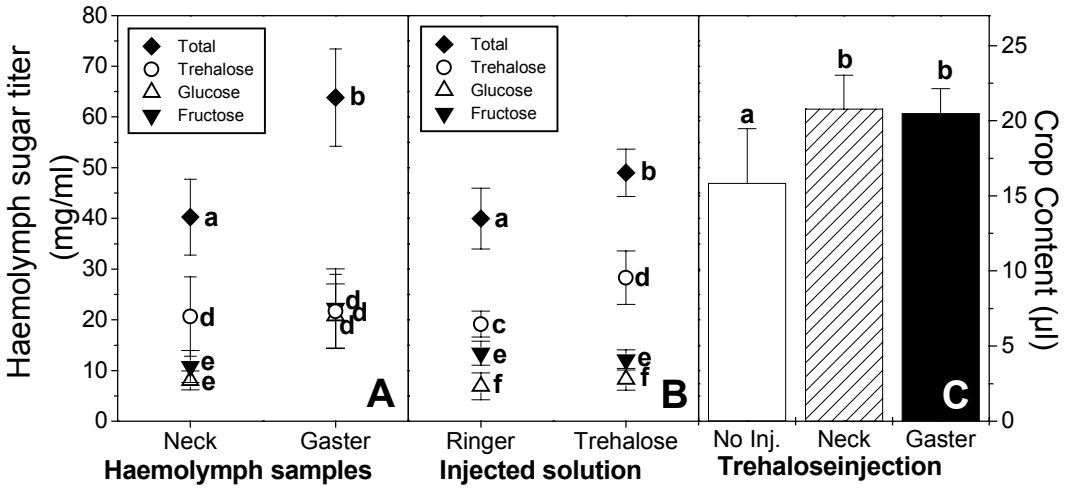

Fig. 19. Preliminary investigations

Fig. 19A. Comparison between haemolymph sugar titers from haemolymph samples taken either from the neck or from the gaster of the same individuals, 15min after feeding (mean ± SD, n = 17). Bees were fed 30µl 30% sucrose solution. Manova, $F_{(3,13)} = 15.21$, $p < 0.0001$; values sharing the same letter are not statistically different (after Newman-Keuls comparison, $\alpha < 0.01$).

Fig. 19B. Haemolymph sugar titers (mean ± SD) of bees injected with 1µl 1M trehalose into the neck membrane 15min after feeding 30µl 30% sucrose solution (n = 10). Dissections were carried out 3min after injection. Bees injected with 1µl Ringer served as controls (n = 10). Manova, $F_{(3,72)} = 10.14$, $p < 0.0001$; values sharing the same letter are not statistically different (after Newman-Keuls comparison, $\alpha < 0.01$).

Fig. 19C. Crop contents (mean ± SD) for bees injected with 1µl 1M trehalose into the neck membrane (n = 8) or gaster (n = 8), 15min after feeding 30µl 30% sucrose solution. Dissections were carried out 15min after injection (i.e., 30min after feeding). Uninjected bees served as controls (n = 12). Manova, $F_{(2,25)} = 9.84$, $p = 0.0007$; values sharing the same letter are not statistically different (after Newman-Keuls comparison, $\alpha < 0.01$).

Haemolymph composition and the control of the proventriculus

In Fig. 20A the crop contents are presented for bees injected with different solutions. Bees were injected 15min after feeding and dissected 15min after injection. All groups had mean metabolic rates of about 6 mlCO_2/h, which means that bees needed approximately 1.85 mg of

sugars (5.5µl of solution) for their metabolism in the period from injection until dissection. The injection with the non-metabolisable sugar sorbose (0.053mol) is expected to raise the haemolymph osmolarity by 10% (Crailsheim, 1985). Independently of the increase in haemolymph osmolarity, bees injected with sorbose had a crop content equal to that of the control bees (c. 16µl), either not injected or injected with Ringer (Fig. 20A, for statistics see figure legend). The injection of 1µl 1M trehalose (0.38mg sugar) led to an equivalent rise in osmolarity as the injection of sorbose, furthermore it doubled the amount of trehalose normally found in the haemolymph. Bees injected with trehalose had a crop content of 21µl, about 5µl (c. 1.7 mg sugar) more than the control bees. As these 1.7 mg sugar approximately correspond to the amount of sugar needed to cover the energy demands after injection (see above), the flow of solution from crop to midgut must have stopped immediately after injection of the trehalose solution. As mentioned above, the injected sugar amount (0.38mg) is only sufficient to cover the observed metabolic needs for about 4 min. The injection of 1µl 2M glucose or 2M fructose raised the haemolymph osmolarity twice as much as the injection of 1µl 1M sorbose or 1M trehalose, but lead to a similar increase in the amount of metabolisable sugars, just like the injection of trehalose. Bees injected with glucose or fructose, both showed crop contents of about 18µl. Thus, the amount transported through the proventriculus was about 3µl lower than that in the control groups, but 2µl higher than that of the trehalose-injected group.

Fig. 20B presents the midgut weights of the same individuals plotted in Fig. 20A. Unexpectedly, bees injected with Ringer have higher midgut weights (c. 12mg) than uninjected bees (c.10mg). This is a general pattern that has been observed in several experiments. For this reason, bees injected with Ringer, rather than untreated bees, are used as controls for comparisons of midgut weights. The midgut weight of bees injected with trehalose was reduced by about 3mg in comparison to bees injected with Ringer, whereas that of bees injected either with glucose, fructose or sorbose was not statistically different from that of bees injected with Ringer (for statistics see figure legend). However, as the sugar concentrations in the midgut were not measured, it can not be argued that the reduction in midgut weight resulted from a lower content of sugars.

Haemolymph sugar titers were similar in all groups. The only exception were bees injected with sorbose (Fig. 20C). Though the injections of trehalose, glucose and fructose should have led to an increase of about 22mg/ml in haemolymph sugar titers for the respective sugars, no increase in the injected substances was detectable 15min after injection. All groups showed trehalose titers of approximately 21mg/ml and glucose and fructose titers of approximately 9mg/ml, respectively. This results in total haemolymph sugar titers of approximately

40mg/ml. Only the sorbose injection led to an obvious increase in the ”fructose” peak, when analysed via HPLC. This is because sorbose has the same running time as fructose, so that the ”fructose” titer presented in Fig. 20C is indeed a mixture of both, fructose and sorbose titers. In comparison to all other investigated groups, bees injected with sorbose did not only differ with respect to the ”fructose” titer, but they also had a slightly lower trehalose titer (17mg/ml). Although the trehalose titer was lower and the glucose titer was equal to the other groups, the total haemolymph sugar titer was much higher in comparison to the control groups due to the increased ”fructose” (sorbose) titer.

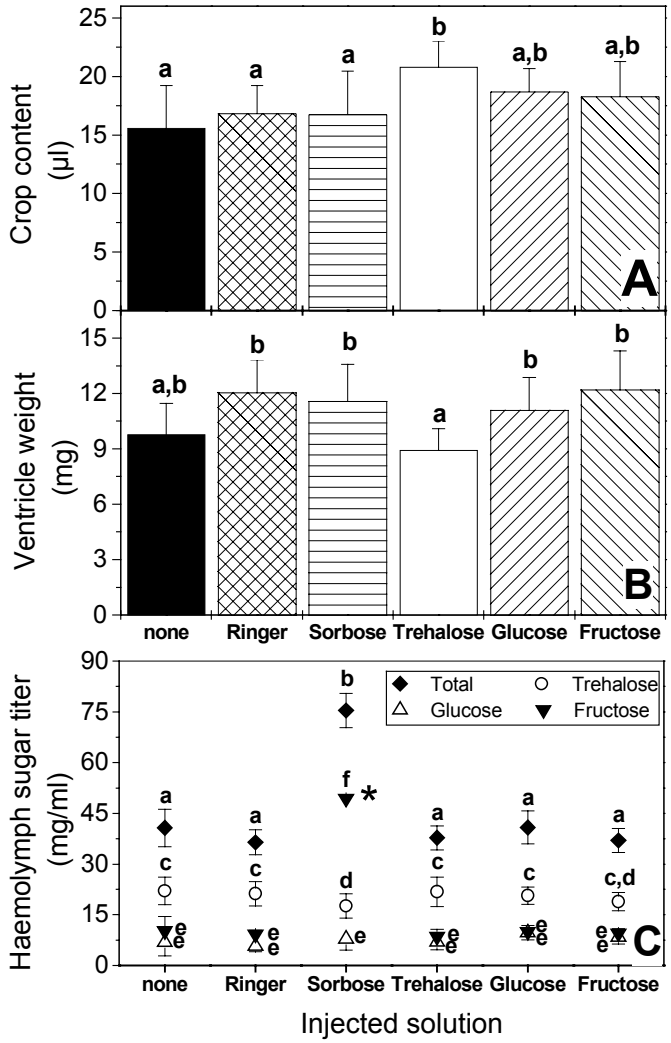

Fig. 20. Crop contents (**A**), ventricle weights (**B**) and haemolymph sugar titers (**C**) (mean ± SD, n = 8 to 13) for bees injected with 1µl of either 1M sorbose, 1M trehalose, 2M glucose and 2M fructose. Untreated bees and bees injected with 1µl Ringer served as controls. Injections were administered 15min after feeding. Dissections were carried out 15min after injection. As all groups had mean metabolic rates between 6.1 to 6.5 $mlCO_2/h$, the metabolic rates of the bees are considered to be similar. **A:** Manova, $F_{(5,47)} = 3.63$, p = 0.007; columns sharing the same letter are not statistically different (after Newmann-Keuls comparison $\alpha < 0.05$); **B:** Manova, $F_{(5,47)} = 4.77$, $p < 0.001$; columns sharing the same letter are not statistically different (Newman-Keuls $\alpha < 0.05$). **C:** The star indicates that this value not only represents the fructose titer, but is a mixture of both, fructose and sorbose titer, as both sugars have the same running time in the HPLC. Manova, $F_{(15,25)} = 71.85$, $p < 0.0001$; values sharing the same letter are not statistically different (after Newmann-Keuls comparison $\alpha < 0.05$).

Time dependence of the proventriculus response to trehalose injections

In order to get further insight into the dynamics of the regulation and to gain knowledge about the velocity at which the observed reactions take place, both the time of trehalose injection after feeding and the period between injection and dissection were varied. Fig. 21A pre-

sents the crop contents of bees injected with 1µl 1M trehalose in comparison to control, uninjected bees. One group was injected 5min after feeding (I), while the other was injected 20min after feeding (II). All bees were dissected 10min after the respective injections, i.e., 15 or 30 min after feeding, depending on the group.

When bees were injected 5min after feeding, mean crop contents (about 22µl) of bees injected with trehalose and control bees were similar (Fig. 21A, I). Surprisingly, bees injected 20min after feeding showed a highly significant difference in the crop contents compared to control bees (Fig. 21A, II; for statistics see figure legend), although the time between injection and dissection was equal to bees injected after 5min (Fig. 21A, I). A crop content of 13µl was found in control bees, whereas 18µl remained in the crop of bees injected with trehalose. This difference of 5µl (c. 1.7 mg sugar) approximately corresponds to the amount of sugars needed to cover the energy demands during 10 minutes (the time from injection to dissection) at the measured mean metabolic rate of 7 mlCO_2/h (c. 1.4 mg sugars). In the same way as already mentioned above, the solution flow from crop to midgut must have stopped immediately after injection of the trehalose solution. However, as for the experiments presented in Fig. 20A, it has to be noted that the amount of injected sugars (0.38 mg) is only sufficient to cover the observed metabolic needs for approximately 3 min.

Fig. 21B presents the haemolymph sugar titers of bees injected at different times after feeding. For both bees injected after 5 minutes and uninjected bees, all single haemolymph sugars were statistically not different (Fig. 21B, I; for statistics see figure legend). However, the slight increase of all single haemolymph sugars led to a significantly higher total haemolymph sugar titer in the trehalose injected bees (48mg/ml), in comparison to the uninjected bees (41mg/ml). When the injection was administered 20min after feeding, however, the total haemolymph sugar titer of the trehalose-injected bees (39 mg/ml) was lower than in the control group (50 mg/ml) (Fig. 21B II). This difference was due to lower glucose and fructose titers, as the trehalose titer was similar in both groups (21mg/ml).

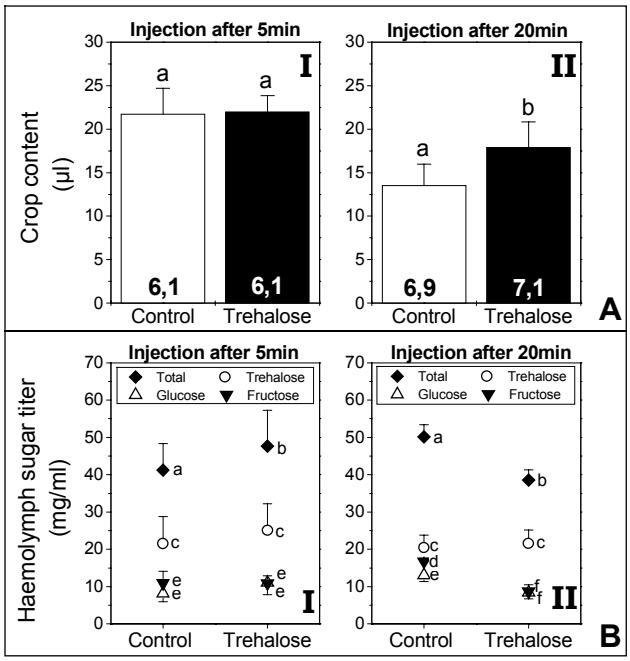

Fig. 21. Both crop content (**A**) and haemolymph sugar titer (**B**) of bees injected at different times after feeding with 1µl 1M trehalose (mean ± SD). Untreated bees served as controls (n = 8 to 22). Bees were fed 30µl 30% sucrose solution. Dissections were carried out 10min after injections. One group was injected 5min after feeding (**I**), while the other was injected 20min after feeding (**II**). Numbers in the columns represent the mean metabolic rate.
Fig. 21A. Crop content (µl). Columns sharing the same letter are not statistically different. Statistics for both time intervals were done separately. **I** (T-test: t = 0.99, p = 0.33); **II** (T-test: t = 3.029, p = 0.009)
Fig. 21B. Haemolymph sugar titers (mg/ml). Statistics for both time intervals were done separately. Values sharing the same letter are not statistically different; (after Newman-Keuls $\alpha < 0.05$). **I** Manova, $F_{(3,13)} = 2.05$, $p = 0.11$; **II** Manova, $F_{(3,60)} = 18.82$, $p < 0.00001$.

Functional questions about the control of the proventriculus

The following investigations were performed to find out why the honeybee proventriculus overregulates under particular experimental conditions. It is assumed that bees engaged in regular and continuous foraging overregulate, because they need an extra amount of sugars for the imminent take-off and flight back to their hive.

In a first experiment, bees trained to collect sugar solution provided by a pump at a very low flow rate were caught before they finished their feeding activity and dissected immediately afterwards. As a consequence, any potential feed-forward regulation of the proventriculus prior take-off should not have still occurred. The pump provided 30% sucrose solution at a flow rate of 2.36µl/min. Under these conditions, the trained bee stayed at the feeding place for 15 to 20min, reaching therefore a crop load of 35.4 to 47.2 µl. Bees were caught after 12.7min feeding, when they had collected exactly 30µl, and were dissected immediately afterwards. Following the assumption that the proventriculus transports a surplus amount of sugar solution immediately before leaving for the hive, the regulation should have been precise under these conditions. However, more solution left the crop than needed to cover the metabolic demands. Even under this conditions a constant surplus of approximately 1mg sugar left the crop (Fig. 22).

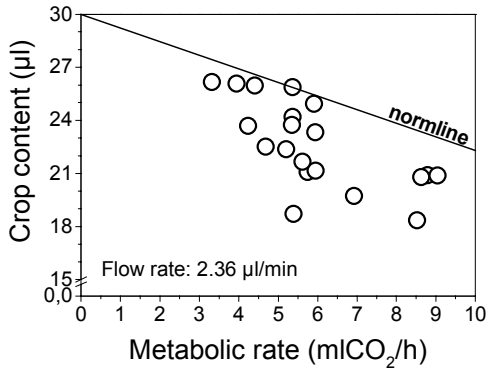

Fig. 22. Crop content(µl) of bees collecting sugar solution provided by a pump at a flow rate of 2.36µl/min, as a function of their metabolic rates. Bees were fed with 30% sucrose solution for 12.7min (i.e., they collected 30µl). The "normline" represents the expected sugar transport rate through the proventriculus, calculated out of the measured CO_2 production.

In two additional experiments, the distance between hive and feeding place was increased from 80 to 1000m, or bees were trained to walk 15 cm to reach the feeding place. Bees flying different distances to arrive at the feeding places had the same crop content of about 16µl 30min after feeding ended (Fig. 23, right, for statistics see figure legend). Bees walking to reach the feeding place also showed a crop amount of 16µl (Fig.23, left). However, the crop content should average 19µl based on the bee's metabolic expenditure (mean: 6 $mlCO_2/h$). This means that under all investigated conditions, the bees passed approximately 3µl more than they needed to cover their metabolic expenditure through the proventriculus.

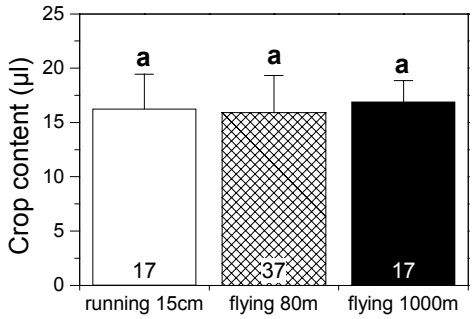

Fig. 23. Crop contents (µl) of bees fed 30µl 30% sucrose solution. One group of bees was trained to run to the feeding place over a distance of 15cm. Foragers of the two other groups were flying over distances of 80m and 1000m, respectively. As mean metabolic rates of the three groups ranged from 5.8 to 6.2 $mlCO_2/h$, they were considered to be similar. Numbers in the columns represent the sample size. No statistical differences were found between the groups (Manova, $F_{(2,68)} = 0.58$, $p = 0.563$).

3.3.3. Discussion

Feedback mechanisms acting on the proventriculus activity

First, it should be commented that it is unclear what exactly caused the collapsing metabolisms of injected bees, which were not accompanied by distinct changes in observable behaviour. Marked bees recovered after one night, and were generally able to forage again showing normal metabolic rates. As (Crailsheim, 1985) emphasised that the mortality of bees injected with water or solution of sodium chloride was the same, and we found mean metabolic rates

of 6.1 mlCO_2/h before and of 3.25 mlCO_2/h after injections in (Roces and Blatt, 1999), it appears that injuries of the bees by injection is a general experimental problem. In our investigations on more than 100 injected bees, less than 5 bees actually died and only about 10% to 20% showed collapsing metabolisms. In this context, it is unknown whether the injury directly influences the crop emptying rates. But it is clear that collapsing metabolisms causes lower mean metabolic rates. As lower metabolic rates lead to both decreased crop emptying rates and changes in haemolymph sugar titers (Blatt and Roces, 2001b), comparisons of bees which have been injured and bees which have not automatically cause inaccuracies and high variability in the results. Therefore bees with varying or collapsing metabolisms were not used in this study.

Bees injected with trehalose, glucose and fructose reduced the transport rates from crop to midgut in comparison to control bees, whereas bees injected with sorbose did not. This supports the conclusion that the nutritive value rather than the osmolarity effects of the injected sugars has an impact on the proventriculus activity. These results correspond well with those of our previous study (Roces and Blatt, 1999), but disagree with those of (Crailsheim, 1988b). Not only (Crailsheim, 1988b), but also (Gelperin, 1966) found reduced crop emptying rates after injections of non-metabolisable sugars. However, as blowflies are non-social insects feeding on a large variety of different foods and have low metabolisms, they are difficult to compare with the highly social honeybees, which mainly feed on sugar solutions and which possess very high metabolic rates.

There are some differences between Crailsheim's study and our work. Crailsheim worked with immobilised bees, injected into the gaster before feeding and did not monitor the metabolic rates of the investigated animals. In contrast, we investigated freely foraging bees, injected into the neck membrane after feeding, and monitored the metabolic expenditure of the bees. In preliminary experiments, we found that working with immobilised bees led to less precise adjustments of crop emptying rates to metabolic rates (unpublished results). As the effect of trehalose injection on the crop emptying rate is independent of the compartment into which injections are administered, the different results from (Crailsheim, 1988b; Roces and Blatt, 1999) and the present investigation can not be attributed to this factor. As injections can lead to deviations in the metabolic rates as discussed above, it can not be excluded that in the study of (Crailsheim, 1988b), the observed differences between the bees injected with non-metabolisable substances and the control groups were due to different metabolic demands. Whether the different times at which injections were administered might causes the different

results found by (Crailsheim, 1988b) in comparison to our results (this study and (Roces and Blatt, 1999)), will be discussed below.

Our results showed that, although the injected amount of trehalose was only sufficient to cover the bees' metabolic demands for 4 min, the proventriculus was closed for about 15min. A possible explanation would be that the bees respond to the high haemolymph trehalose titer by closing the proventriculus, but that the sugars which have already entered the midgut continue to diffuse into the haemolymph, as the transport from midgut to haemolymph is passive (Crailsheim, 1988a). If one follows this assumption, there should be smaller amounts of sugar in the midgut for bees injected with trehalose in comparison to the control bees. Unfortunately we were not able to measure the total amounts of sugar in the midgut, but the lower midgut weight of trehalose injected bees in comparison to bees injected with Ringer is at least a powerful indication that after the trehalose injection, the bees supported their metabolic demands with sugars from the midgut. As indicated in the results, bees injected with Ringer were used as controls during comparisons of midgut weights. It is not clear why injected bees showed larger midgut weights than uninjected bees. Since we previously found lower rectum contents after injections in bees (Roces and Blatt, 1999), it could be assumed that for unknown reasons, the treatment slowed down both the solution transport rate from midgut into the haemolymph and the urine production rate.

Though the nutritive value is almost equal, the injection of 2M glucose or 2M fructose caused sugar transport rates that were not as reduced as the sugar transport rates after injections of 1M trehalose. Unlike trehalose, glucose and fructose are not only present in the haemolymph, but also in the midgut. Therefore the injection of glucose and fructose caused sugar titers in the haemolymph almost as high as the respective titers in the midgut (30-35mg/ml, unpublished results), so that no passive diffusion from midgut to haemolymph is possible until the monosaccharides are transformed into trehalose. If the proventriculus activity is influenced only by the trehalose titer and not by other haemolymph sugars, as we assume, the invariant midgut weights in bees injected with the monosaccharides become understandable. In contrast to these results in a previous study we found that injections of 1.5 M glucose and 0.75 M trehalose led to the same decrease in sugar transport rates (Roces and Blatt, 1999). But as (Roces and Blatt, 1999) found mean metabolic rates of 6.1 mlCO_2/h before injections and mean metabolic rates of 3.25 mlCO_2/h after injections and as the measurements of metabolic rates and crop emptying rates were not performed with identical individuals, possible metabolic differences between the groups may have led to imprecise results.

Though the injections had clear effects on the crop emptying rates and on the midgut weights, no effects on the haemolymph sugar titers were detectable 15min after injection, with the one exception of bees injected with sorbose. As the injection of trehalose, glucose and fructose is expected to cause very high haemolymph sugar titers, one could suggest that either the injected sugars did not arrive in the haemolymph or that they must have been metabolised at the time of dissection. Not only reduced crop emptying rates were observed after injection, but also bees injected with trehalose still had higher trehalose titers 3min after injection than bees injected with Ringer. As a result, we are confident that the injected substances actually reached the haemolymph, supporting the assumption that the injected sugars were already metabolised when the haemolymph samples were taken.

As it has been shown that haemolymph sugar titers differ when samples are taken from neck or gaster, even if they are taken from the same individuals, the haemolymph sugar titers of the present investigation were only compared with the results of those authors which also took haemolymph samples from the neck membrane. The haemolymph sugar titers of the present investigation were similar to those reported by (Arslan, et al., 1986; Bozic and Woodring, 1997; Woodring, et al., 1993; Woodring, et al., 1994).

As sorbose can not be metabolised by bees (Vogel, 1931), a high titer was found in the haemolymph even 15min after injection. However, the shown titer can only roughly represent the sorbose titer, as it was not possible to distinguish between sorbose and fructose via HPLC. The extraordinarily high ”sorbose” titer might have obstructed the conversion of glucose and fructose to trehalose, leading to the slightly lower trehalose titer in the haemolymph of the bees injected with sorbose in comparison to all other groups.

Time dependence of the proventriculus response to trehalose injections

Bees injected 20min after feeding and dissected 10min later responded to the trehalose injection by reducing their crop emptying rates. As (Crailsheim, 1985) found that the mixing of injected substances is already complete after 15 sec at temperatures of 22-25°C, it is not surprising that a reduced sugar transport rate from crop to midgut was detectable, even when the dissection was performed already 10min after injection. Furthermore it was predictable that the injected amount of trehalose would be not detectable any more 10min after injection, as bees with the observed metabolic rates (7 mlCO_2/h) consume the whole amount of injected trehalose in at least 4min.

But it is very astonishing that bees injected 5min after feeding (and not 20min, as described above) and dissected 10min later did not reduce their crop emptying rates, though the condi-

tions (training, feeding solution, handling, time to respond to the injection) were otherwise the same. As only a slight increase in haemolymph sugar titers was found for the bees injected with trehalose, it is unclear where the surplus of sugars remained and why the proventriculus was not influenced.

With regard to the injection of non-metabolisable sugars, (Crailsheim, 1988b) showed reduced sugar transport rates after sorbose injections 2min before feeding. We never found reduced sugar transport rates after injection, neither in the present investigation, nor in (Roces and Blatt, 1999). Our injections were always carried out after feeding, so that it can not be excluded that the different results may have to be put down to the different times at which injections were performed. However, comparisons are difficult because he worked with immobilised bees and no measurements of their metabolic rates were performed.

Functional questions

A constant overregulation was observed, no matter whether the bees were still collecting solution at the feeder, or whether they had to fly a long distance or walk a short one to reach the feeding place. The observed overregulation was very stable and of similar magnitude. We were unable to demonstrate that the overregulation is the result of feed-forward regulation for the imminent take-off and flight. It is therefore conceivable that this phenomenon is a feature of the system regulating the proventriculus activity that can not be modulated. Both the invariance in the pattern of overregulation and the lack of reaction to trehalose injection briefly after feeding may indicate that the regulatory system works initially in an open-loop condition without feedback, for unknown reasons. However, these are only conjectures that need to be investigated in further experiments.

3.4. Haemolymph sugar titers of honeybees feeding on natural food sources and the occurrence of sucrose in the honeybee haemolymph

3.4.1. Introduction

Sugars are the major source of energy in honeybees (Rothe and Nachtigall, 1989; Sacktor, 1970). Sugars are obtained by the collection of nectar and honey dew. The gathered food is partly used for honey production within the hive and partly for supplying the bees' own metabolism. The proventriculus regulates food passage from crop to midgut (Bailey, 1952; Blatt and Roces, 2001a; Crailsheim, 1988b; Peng and Marston, 1986; Roces and Blatt, 1999). In the midgut, sucrose is digested by an α-glucosidase (Bounias and Morgan, 1984a). Fructose and glucose are quickly transported out of the midgut into the haemolymph (Crailsheim, 1988a; Crailsheim, 1988b). Haemolymph glucose is taken up by fat body cells, which synthesise the disaccharide trehalose and release it into the haemolymph to be used as a source of energy in all other organs (Beenakkers, et al., 1985). In insects that metabolise sugars as their major source of energy, trehalose is the major fuel present in the haemolymph (Candy, et al., 1997).

There have been various investigations on the haemolymph sugar titers, all of which were carried out under different experimental conditions. Haemolymph trehalose titers from 2 mg/ml (Bounias and Morgan, 1984c) to 40 mg/ml (Bozic and Woodring, 1997), and glucose and fructose titers from 2 mg/ml (Abou-Seif, et al., 1993) to about 15 mg/ml (Fell, 1990; Leta, et al., 1996) have been measured. The haemolymph sugar titers in bees have been reported to change in response to fed solution (Crailsheim, 1988b), season (Bozic and Woodring, 1997; Crailsheim, 1988b), and behavioural pattern (Bozic and Woodring, 1997). Based on this high variability, it has been suggested that there is no need for haemolymph sugar homeostasis in insects (Candy, et al., 1997), even though regulating hormones have been found in the honeybee (Gäde, 1996). More recently we have shown that trehalose titers were unaffected by the time after feeding, the accuracy of regulation, and the concentration of fed sugar solution (Blatt and Roces, 2001a). Therefore we suggested that the trehalose titer is regulated in the honeybee and represents the controlled variable in the feedback loop responsible for the regulation of the proventriculus.

Under natural conditions, honeybee foragers collect nectars that differ in both their sugar concentration and composition. Besides, bees not always return to the hive with a filled crop, so that the partial crop-filling may influence the regulation of the proventriculus activity and therefore the haemolymph sugar titers. The aim of the present study was to investigate

whether regulated haemolymph sugar titers are also observed in honeybee foragers collecting nectar from natural food sources, and whether the haemolymph sugars correspond with the predictions based on the results obtained with trained honeybees foraging at artificial food sources (Blatt and Roces, 2001a). To achieve this, crop contents and haemolymph sugar titers of foraging honeybees returning from natural food sources were investigated over the whole foraging season, from May to August.

In preliminary measurements we detected sucrose in the haemolymph of foragers returning from natural food sources. The occurrence of sucrose in the honeybee haemolymph has been previously reported by Abou-Seif et al. (1993), Bounias and Morgan (1984c), Geisler and Steche (1963), Leta et al. (1996) and Maurizio (1965), but Arslan et al. (1986), Beutler (1937) and Woodring et al. (1993), in contrast, have never found sucrose during their investigations. As colleagues from both groups assume that the detected haemolymph sucrose is a result of contamination, the second part of this study deals with the question whether sucrose indeed occurs in the honeybee haemolymph or if it represents an experimental contamination.

3.4.2. Results

Bees collecting small, medium or large amounts of nectar showed similar haemolymph sugar concentrations (Fig.24A). Independently of the amount carried in the crop, foragers showed mean trehalose titers of approximately 15 mg/ml. Mean titers of glucose and fructose (values between 6.1 – 8.9 mg/ml) were observed to be statistically similar. Aside from trehalose, glucose and fructose also small amounts of sucrose (approximately 1.5 mg/ml) were found in the haemolymph. Therefore mean total haemolymph sugar titers reached approximately 32 mg/ml, independently of the amount carried in the crop.

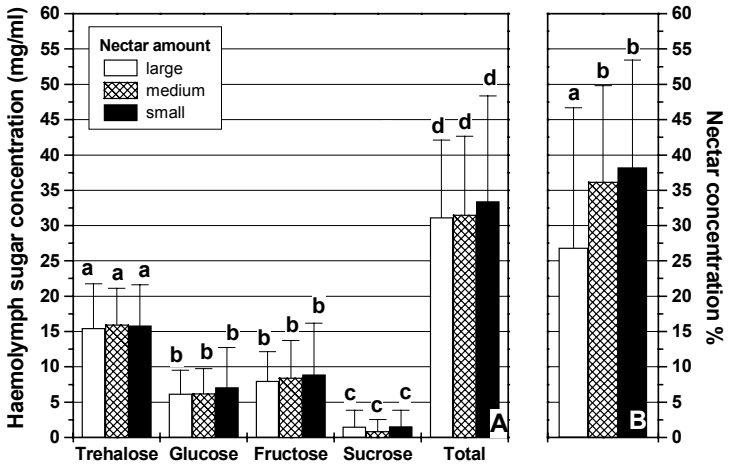

Fig. 24. Relationship between the amount of nectar in the crop of bees returning from foraging trips and the haemolymph sugar concentration of the foragers (**A**). MANOVA $F_{8,61} = 0.20$, $P<0.99$; columns sharing the same letter are not statistically significant after Newman-Keuls comparisons, $P<0.05$. The relationship between the amount of nectar in the crop and the concentration of the collected nectar (**B**). MANOVA $F_{2,12} = 5.01$, $P<0.008$; columns sharing the same letter are not statistically significant after Newman-Keuls comparisons, $P<0.05$. Amounts are grouped as small (N=34), medium (N=58) and large (N=34).

Mean sugar concentration of crop content averaged 37% for foragers carrying small or medium amounts in their crops, whereas that for foragers carrying large amounts were significantly lower (26.8%).

Fig.25 shows the relationship between haemolymph sugar titers and the date of investigation. In spite of high variations, haemolymph sugar titers of glucose, fructose and sucrose were observed to decrease significantly with season (for statistics see figure legend). In contrast, there was no relationship between season and trehalose titers (15.8 ± 5.5 mg/ml). The patterns exhibited by the titers of trehalose, glucose, fructose and sucrose resulted in decreasing total haemolymph sugar concentrations over the season. Total haemolymph sugar concentrations decreased from approximately 40 mg/ml at the beginning of May, to approximately 23 mg/ml at the end of August.

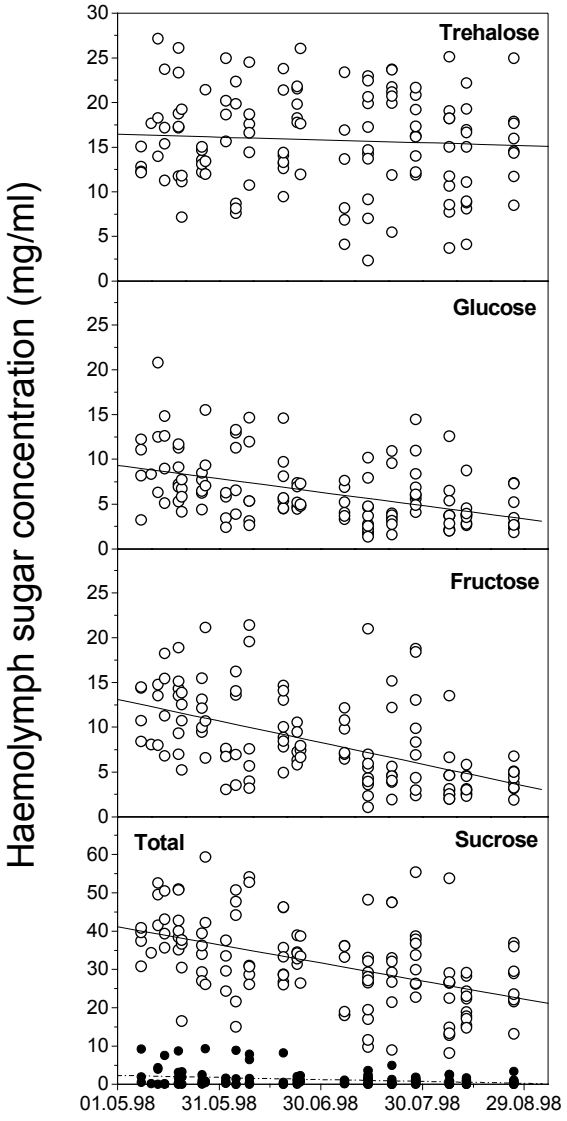

Fig. 25. Haemolymph sugar level *versus* season. Trehalose levels C_{Tre} remained constant over season *D*. (C_{Tre}=*16.72, N*=124, *P*=0.48). Glucose levels C_{Glu} decreased significantly over season *D*.(C_{Glu}=9.51-0.05**D*; *N*=124, *r*=-0.46, *P*<0.0001). Fructose levels C_{Fru} decreased significantly over season *D*.(C_{Fru}=13.43-0.08**D*; *N*=124, *r*=-0.54, *P*<0.0001). Sucrose levels C_{Suc} decreased significantly over season *D*.(C_{Suc}=2.39-0.02**D*; *N*=124, *r*=-0.28, *P*<0.002). Total haemolymph sugar levels C_{Tot} decreased significantly over season *D*. (C_{Tot}=41.81-0.16**D*; *N*=124, *r*=-0.48, *P*<0.0001).

Foragers returning from natural food sources carried solutions with concentrations ranging from 9 - 66% sugar (Fig. 26). In addition five bees, which had collected water were recorded. Even though the variations are high, a strong negative relationship between nectar concentration and season was detected (for statistics see figure legend). While bees foraging in the beginning of May collected on average solutions with more than 40% sugars, bees foraging in the end of August collected solutions with sugar concentrations lower than 30%.

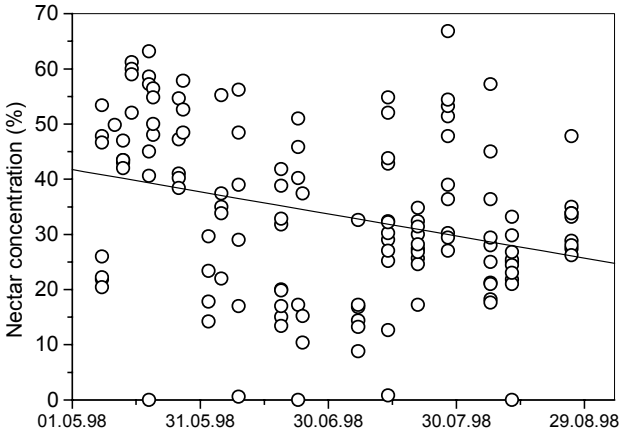

Fig. 26. Nectar concentration found in the foragers' crop *versus* season. Nectar concentration C_{Nec} decreased significantly over season D. (C_{Nec}=-0.133**D*; N=134, r=-0.30, P<0.0004).

Fig. 27 shows the relationship between sugar concentration of the carried nectar and haemolymph sugar titers for the same foragers. In spite of high variability, haemolymph sugar titers of glucose, fructose and sucrose were observed to increase significantly with increasing sugar concentration of the carried nectar. Even when most bees had, independently of the nectar concentration, only small or no amounts of sucrose in the haemolymph (in average 1.3±2.1mg/ml), the likelihood of the occurrence of sucrose increased with increasing crop nectar concentration. Nevertheless, the sucrose concentrations in the haemolymph did sometimes reach rather high values of up to 9.3 mg/ml. In contrast to glucose, fructose and sucrose, no relationship between crop nectar concentrations and haemolymph trehalose titers was observed. The patterns exhibited by titers of trehalose, glucose, fructose and sucrose resulted in increasing total haemolymph sugar titers with increasing nectar concentrations. Total haemolymph sugar concentrations increased from approximately 22 mg/ml for diluted to approximately 45 mg/ml for the most concentrated nectars.

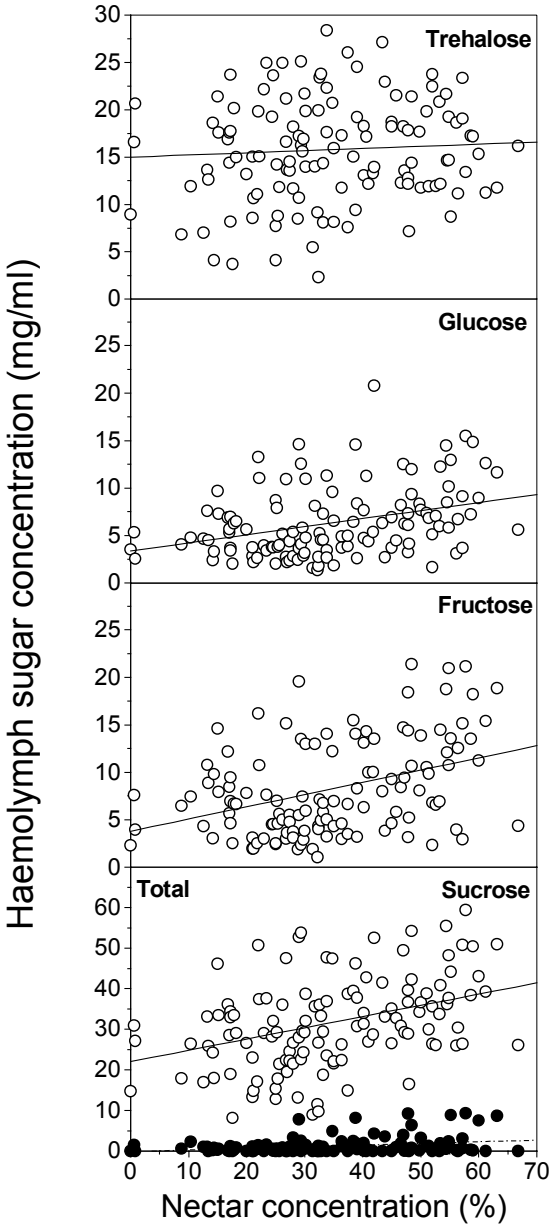

Fig. 27. Haemolymph sugar level *versus* nectar concentration found in the foragers' crop. Trehalose levels C_{Tre} remained constant over season *D*. (C_{Tre}=*15.00,* N=124, P=0.48). Glucose levels C_{Glu} increased significantly over season *D*. (C_{Glu}=3.37+0.085**D*; N=124, r=0.36, P<0.0001). Fructose levels C_{Fru} increased significantly over season *D*. (C_{Fru}=3.80+0.128**D*; N=124, r=0.40, P<0.0001). Sucrose levels C_{Suc} increased significantly over season *D*. (C_{Suc}=-0.12+0.040**D*; N=124, r=0.30, P<0.0007). Total haemolymph sugar levels C_{Tot} increased significantly over season *D*. (C_{Tot}=22.06+0.277**D*; N=124, r=0.38, P<0.0001).

Occurrence of sucrose in the haemolymph

In order to test whether the sucrose peak obtained by HPLC-analysis really represents sucrose, haemolymph samples, were divided into two halves. One part was treated with the enzyme Invertase, which splits sucrose into glucose and fructose, while the other part served as control. Fig. 28. shows the glucose, fructose and sucrose concentrations of the divided haemolymph samples. While 3.06 mg/ml sucrose was detected in the control, no sucrose was left in those samples, which were treated with Invertase. In addition the glucose and fructose peaks of the samples treated with Invertase increased by approximately 1.5mg/ml in comparison to

the controls. Therefore the peak obtained by HPLC-analysis is with almost absolute certainty sucorse.

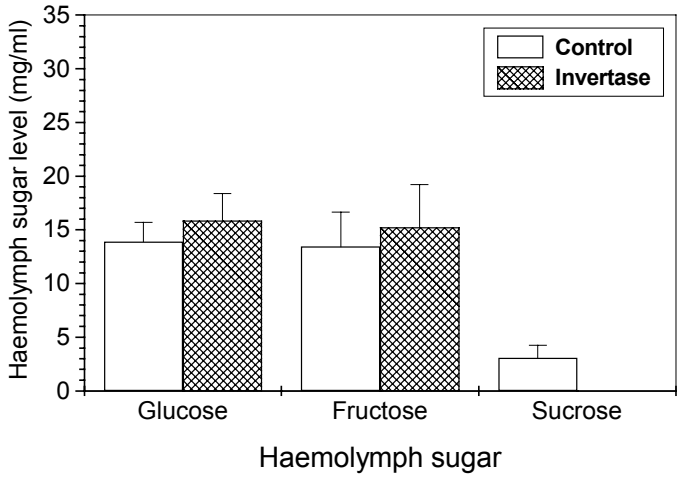

Fig. 28. Glucose, fructose and sucrose concentrations (mean ± SD) of four haemolymph samples, which were taken immediately after bees collected 50µl 50% sucrose solutions. Samples were divided into two halves. In one half 1µl sucrose splitting Invertase was added, the other half served as control into which 1µl distillated water was added. Both were kept for 30min at 45°C before analysis.

The aim of the next experiment was to investigate, in which compartment the splitting of sucrose occurs. It is assumed that the occurrence of sucrose in the haemolymph is much more likely a contamination, if the sucrose is already totally split to glucose and fructose in the crop. Therefore the crop and midgut concentrations of sucrose, glucose and fructose were determined. Fig. 29A. shows the relationship between the crop sugar titers and time after feeding. Bees fed 30% sucrose solution had almost only sucrose and very small amounts of glucose and fructose in the crop directly after feeding. The concentration of sucrose decreased, while the concentrations of glucose and fructose increased at the same rate with time. However, most of the sucrose (249.8 mg/ml) was still not split even 90min after feeding, so that the proportion of glucose (54.5 mg/ml) and fructose (49.5 mg/ml) was much smaller in relation to sucrose.

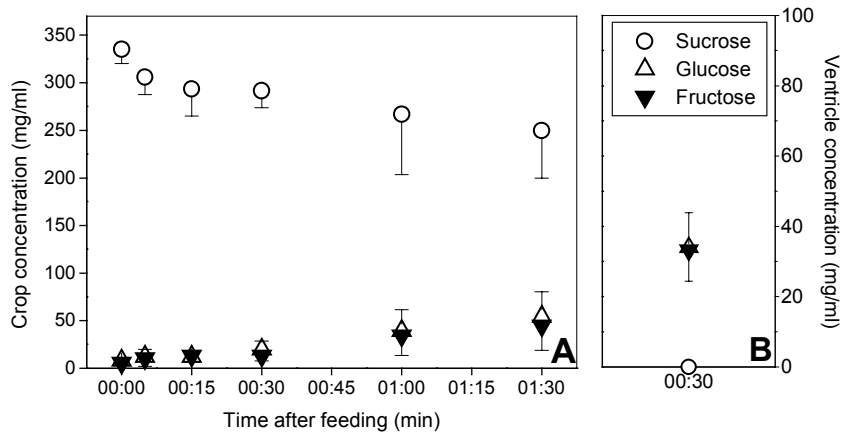

Fig. 29. Relationship between crop sugar concentrations (mean ± SD) and time after feeding. (**A**) Bees were fed 50µl 30% sucrose solutions (338mg/ml); samples were taken 0, 5, 15, 30, 60 and 90min after feeding ended. (**B**) Concentrations of glucose, fructose and sucrose in the midgut 30min after feeding ended of bees fed with 30% sucrose solution.

In the midgut of the honeybees only glucose (33.96±9.56mg/ml) and fructose (33.11±10.81mg/ml), but no sucrose (0.00±0.00mg/ml) was detectable 30 min after feeding 30% sucrose solution (Fig. 29B). Therefore it can be concluded that the splitting of sucrose mainly occurs in the midgut.

It has been argued that even in caged bees fed high concentrated sucrose solutions for a long time no haemolymph sucrose could be detected (Woodring, et al., 1993). Therefore we repeated those experiments, but in addition investigated the metabolic rates of the individuals. The bees were kept in wooden cages for 48h with 50% or 2M (55%) sucrose solution *ad libitum* (Fig. 30A). In bees fed 50% sucrose solution a haemolymph sucrose titer of 0.13±0.28mg/ml, in bees fed 2M sucrose solution no sucrose at all was observed. While foragers showed metabolic rates of approximately 6-7 mlCO2/h, bees kept in cages reduced their metabolic rates to 0.32 mlCO_2/h (inset).

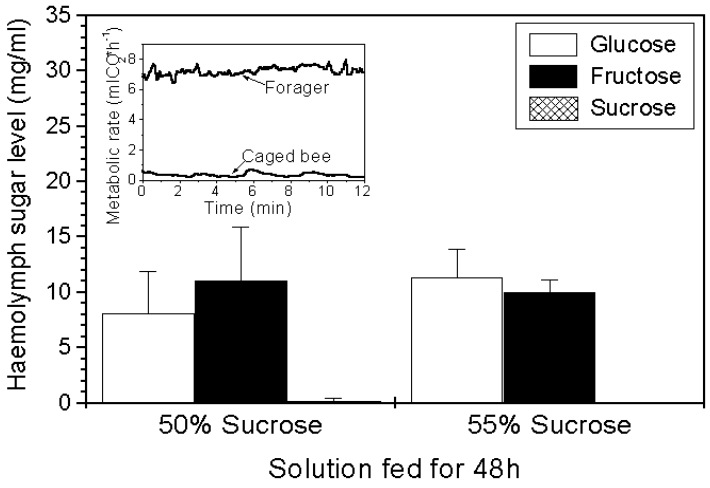

Fig. 30. Glucose, fructose and sucrose levels (mean ± SD) of bees fed either 50% (*N*=11) or 2M (55%) (*N*=10) sucrose solution (A). Bees in groups of ten were kept in small cages under normal day light conditions for 48h with the feeding solutions and water *ad libitum*. The small inset presents examples of metabolic rates for both, a normal forager and a caged bee.

Built on the last experiment in the next experiment it was investigated whether the occurrence of sucrose in the haemolymph is dependent on the metabolic rate of the bees. Fig. 31. shows the relationship between metabolic rates and haemolymph titers of glucose, fructose and sucrose. Haemolymph samples were taken 30min after feeding with 30µl 50% sucrose solution. The concentrations of all three sugars increased significantly with increasing metabolic rates. Sucrose concentrations were generally low (maximum of 2.23mg/ml) and increased with increasing metabolic rates, even though it was sometimes not detected in bees with metabolic rates up to 8 mlCO_2/h.

In the last experiment it was tested whether the way of the sucrose from crop *via* midgut into the haemolymph is so quick that not all the sucrose can be split in the midgut. In order to investigate the speed of solution flow from crop to haemolymph, bees that foraged on 30% glucose solution over a long period were fed 30µl 30% sucrose solution only once, and

haemolymph samples were taken immediately afterwards. The rationale of this procedure is the following: if the collected sucrose solution has left the crop, been cleavaged in the midgut and the products glucose and fructose diffused into the haemolymph during the short time from intake to haemolymph sampling (maximally 90sec), fructose will show up in the haemolymph.

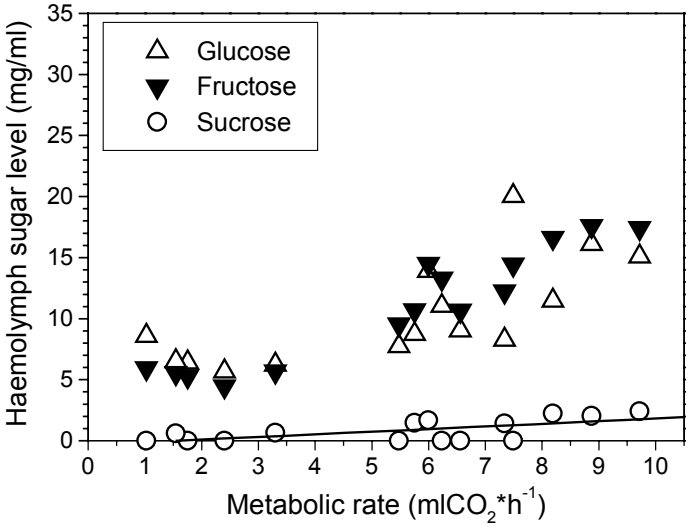

Fig. 31. Glucose, fructose and sucrose levels *versus* metabolic rate V_{CO2} of bees fed 30µl 50% sucrose solution. Haemolymph samples were taken 30 min after feeding ended. Sucrose levels C_{Suc} increased significantly over metabolic rate V_{CO2}. (C_{Suc}=-0.34+0.216*V_{CO2}; N=15, r=0.65, P<0.01).

Both, bees trained and fed 30% sucrose solution and those trained and fed 30% glucose solution served as controls. Bees trained on glucose but fed once with sucrose have similar mean haemolymph fructose titers (9.6±2.9 mg/ml) to bees fed and trained on sucrose (11.8±3.5 mg/ml) (Fig. 32). In contrast, bees trained and fed glucose solutions had only very small fructose titers (2.7±2.3mg/ml). This allows the conclusion that the sucrose transport from crop to midgut, the cleavage into glucose and fructose and the diffusion into the haemolymph does not take longer than 90 sec.

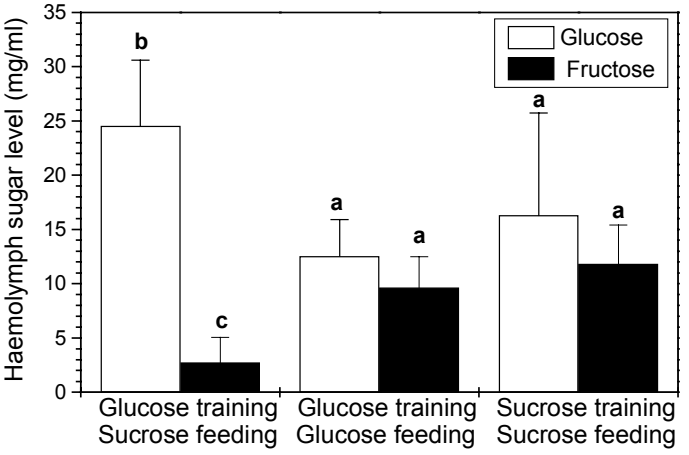

Fig. 32. Glucose and fructose levels (mean ± SD) of bees trained on 30% glucose solutions, which were fed 30% sucrose solution only once, immediately before haemolymph samples were taken (N=9). Both, bees trained and fed 30% sucrose solution (N=6) and bees trained and fed 30% glucose solution (N=5) served as controls. Columns sharing the same letter are not significant different; Manova, $F_{(2,34)}$=12.81, P<0.0001; after Newman-Keuls P<0.05.

3.4.3. Discussion

Nectar concentration

Bees foraging on natural food sources in the surroundings of the bee station collected nectar with concentrations of 9 to 66%. Only few bees were collecting water. These results correspond well the findings of Seeley (1995), who found that bees collected nectars with concentrations ranging between 12 and 65%.

Bees collected lower amounts, if nectar concentrations were high. As it is known that crop filling increases with increasing concentration, increasing flow rate and increasing distance between hive and food source (Núñez and Giurfa, 1996), a possible explanations would be that those flowers offering high concentrated nectar were either growing near to the hive, or presented their nectar with low flow rates. It seems very unlikely that all flowers offering high nectar concentrations were growing near the hive. But it is conceivable that flowers either present high concentrated nectar with lower flow rates or they present diluted nectar with higher flow rates, as they must provide sufficient reward to attract foragers, but they must limit this reward so that the pollinators will go on to visit other plants of the same species (Heinrich and Raven, 1972).

Haemolymph sugar concentration

Quantitative analysis of haemolymph sugars by HPLC confirmed that trehalose, glucose and fructose are the main haemolymph sugars in foraging bees, in concentrations which agree with previous reports (Abou-Seif, et al., 1993; Arslan, et al., 1986; Blatt and Roces, 2001c; Bounias and Morgan, 1984b; Bozic and Woodring, 1997; Fell, 1990; Leta, et al., 1996; Woodring, et al., 1993; Woodring, et al., 1994). However, though concentrations are all more or less in the same range, variations are high. Total haemolymph sugar titers of approximately 20mg/ml (Arslan, et al., 1986) to more than 55mg/ml (Bozic and Woodring, 1997) were reported. Those variances might base upon different handlings. In a previous investigation we were able to show that haemolymph sugar titers were about 30% higher when haemolymph samples were taken from the gaster instead of the neck(Blatt and Roces, 2001b). Furthermore, we were able to show that sugar compositions of fed solutions influence total haemolymph sugar titers (Blatt and Roces, 2001a). The mean total haemolymph sugar titer of 31.5 ± 11.0 mg/ml of honeybees feeding on natural food sources ranks at the lower end of observed total haemolymph sugar titers, even for samples taken from the neck (Blatt and Roces, 2001c; Bozic and Woodring, 1997; Woodring, et al., 1993; Woodring, et al., 1994). As variations are

high and we know nothing about the sugar composition of the collected nectar, we can not answer, why total haemolymph sugar titers of bees foraging on natural food sources are comparatively low.

In a previous study we showed that the composition of haemolymph sugars was dependent on the metabolic rate (Blatt and Roces, 2001c). Based on these data we would estimate that metabolic rates of in the present study investigated bees were higher than 7ml CO_2*h^{-1}. These values are in the range of metabolic expenditures reported for flying honeybees (Balderrama, et al., 1992)

In good agreement with Leta et al. (1996), no relationship between amount of crop content and haemolymph sugar titers were observed.

Glucose, fructose and sucrose concentrations decreased from beginning of May to end of August, while trehalose concentrations were not related to the season. This results in decreasing total haemolymph sugar titers with season. Similar results were found by Bozic and Woodring (1997), who investigated that glucose titers were lower in autumn than in spring. However, as nectar concentrations collected by the bees decreased from beginning of Mai to end of August, the total haemolymph sugar titers are likely to be determined by the nectar concentration. Thus, glucose, fructose and sucrose concentrations increased with increasing concentration of the crop solution and trehalose concentrations stayed constant independently of the nectar concentration in the crop. These results correspond well with those of Crailsheim (1988b) who found for caged honeybees that individuals fed higher concentrated sugar solutions showed higher glucose haemolymph titers. In Blatt and Roces (2001a) the trehalose titer was shown to be unaffected by the time after feeding, the accuracy of regulation, and the concentration of fed sugar solution. Based on that, we suggested that the trehalose titer is regulated in the honeybee. The findings of the present study support this assumption as, unlike all other haemolymph sugars, the trehalose titer remained unaffected by the solution concentration in the crop.

Occurrence of sucrose

Aside from trehalose, glucose and fructose, sucrose was also measured in the haemolymph of foraging honeybees. The occurrence of sucrose in the honeybee haemolymph is still controversially discussed. While Abou-Seif et al. (1993), Bounias and Morgan (1984c), Geisler and Steche (1963), Leta et al. (1996) and Maurizio (1965) found sucrose, Arslan et al. (1986), Beutler (1937) and Woodring et al. (1993) did not, and in several other studies the occurrence of sucrose was not evaluated when investigating bee haemolymph sugar titers (Blatt and Ro-

ces, 2001c; Bounias, 1981; Bounias and Morgan, 1984b; Bozic and Woodring, 1997; Crailsheim, 1988b; Czoppelt and Rembold, 1970; Fell, 1990; Kunert and Crailsheim, 1988). The peak detected by HPLC in our study can be regarded as sucrose, since the fraction was split by the enzyme Invertase. Even though the possibility that the sucrose titers resulted from an injured oesophagus during sampling can not be ruled out, this seems very unlikely. The oesophagus is situated ventrally and haemolymph samples were collected dorsally. In addition, as the oesophagus is small and flexible, it would also rather slip aside if touched by the needle. When the oesophagus was deliberately injured, very high concentrations of sucrose, orginiated from the highly-concentrated nectar contained in the oesophagus, were measured in the haemolymph (unpublished results).

The present results suggest that the occurrence of sucrose in the haemolymph is dependent on the bees' metabolic rates. This conclusion is based on two observations. Firstly, caged bees showing very low metabolic rates in comparison to foragers had almost no sucrose in their haemolymph, though they were fed very high concentrated sucrose solutions. Secondly, a clear correlation between haemolymph sucrose titers and metabolic rates was found for bees fed 50% sucrose solution.

With regard to the absence of sucrose in the honeybee haemolymph, Woodring et al. (1993) has not detected sucrose in caged bees fed 2M sucrose solutions for long periods. Working with two-week old hive bees, which probably have rather low metabolic rates, Arslan, et al. (1986) stated that they have not found sucrose, thus indicating the absence of contamination from the gut. Leta et al. (1996) investigated haemolymph sugar titers in bees preparing to swarm. In a few samples of swarm bees they found very small haemolymph sucrose titers. They explained these findings as gut contamination, as well. Only Abou-Seif et al. (1993) and Bounias and Morgan (1984c) measured sucrose in the haemolymph of the honeybee and did not interpreted their findings as contamination. However, Bounias and Morgan (1984c) reported the collection of 10-20µl haemolymph per bee over a period of 15 days and found haemolymph sucrose titers (up to 89mg/ml) always higher than trehalose, glucose and fructose titers. Because of the extremely large haemolymph amounts collected (Crailsheim (1988b) found that bees have a haemolymph volume of approximately 18µl.), their results are most probably due to contamination. Abou-Seif et al. (1993) found minimal amounts of sucrose in the haemolymph after feeding following prolonged fasting. They discuss that monosaccharides are directly absorbed from the intestine, while oligosaccharides undergo hydrolysis first and that the bees' energy requirements after several hours fasting are possibly so high that so much food is brought to the intestine so that not all oligosaccharides can be split. This is exactly the same explanation that we would give for our findings,

is exactly the same explanation that we would give for our findings, particularly as we were able to show that ingested sucrose occurs in the haemolymph as fructose only after 90 seconds.

There are also indirect indication that the occurrence of sucrose in the haemolymph of the honeybees is no contamination. Bounias and Morgan (1981a), Bounias and Morgan (1981b) and Bounias and Morgan (1984a) found a sucrose splitting glucosidase in the haemolymph of the honeybee and exhausted bees injected with sucrose into the haemolymph are able to fly, even if they needed more pauses than sucrose fed bees (Loh and Heran, 1970). It seems unlikely that bees are physiologically adapted to sucrose splitting in the haemolymph when under natural conditions sucrose never reaches the haemolymph. Nevertheless is sucrose in comparison to glucose, fructose and trehalose a minor haemolymp sugar.

4. LITERATURE

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SUMMARY

The proventriculus regulates the food passage from crop to midgut. As digestion first occurs in the midgut, everything that is left in the crop can be used as food for nestmates and brood. In honeybees, the proventriculus activity therefore needs to be regulated to balance between supporting the single forager with sufficient energy and keeping as much of the collected nectar as possible for the colony. Body reserves are unlikely to be involved in the controlling mechanisms, because honeybees possess only negligible amounts of them. Foragers lose energy via their own metabolism but gain energy from the collected nectar. Therefore it is assumed that these are the two main factors which have an effect on the proventriculus activity. As the haemolymph provides a constantly updated indication of an insect's nutritional state, it is assumed that the factor controlling the proventriculus activity is to be found in the haemolymph.

The purpose of this doctoral thesis was to investigate how output (metabolic rate), input (food quality and food quantity) and internal state variables (haemolymph osmolarity and haemolymph sugar titer) affect each other and which of these factors controls the activity of the proventriculus in the honeybee. Therefore free-flying foragers were trained to collect controlled amounts of different sugar solutions. Immediately after feeding, metabolic rates were measured over different periods of time, then crop-emptying rates and haemolymph sugar titers were measured for the same individual bees.

Under all investigated conditions, both the sugar transport rates through the proventriculus and the haemolyph sugar titers depended mainly on the metabolism.

For bees collecting controlled amounts of 15%, 30% or 50% sucrose solution haemolymph trehalose, glucose and fructose titers were constant for metabolic rates from 0 to 4.5 mlCO_2/h. At higher metabolic rates, trehalose concentration decreased while that of glucose and fructose increased with the exception of bees fed 15% sucrose solution. As the supply of sugar from the crop via the proventriculus was sufficient to support even the highest metabolic rates, the observed pattern must result from an upper limit in the capacity of the fat body to synthesise trehalose. The maximal rate of conversion of glucose to trehalose in the fat body was therefore calculated to average 92.4 µg glucose/min. However, for bees fed 15% sucrose solution both the rate of conversion of glucose to trehalose and the rate of sugar transport from the crop to the midgut were limited, causing an overall decrease in total haemolymph sugar titers for metabolic rates higher than 5 mlCO_2/h. Haemolymph sucrose titers were gen-

erally low but increased with increasing metabolic rates, even though sucrose was not always detected in bees with high metabolic rates.

Though foragers were able to adjust their sugar transport rates precisely to their metabolic rates, a fixed surplus of sugars was transported through the proventriculus under specific feeding conditions. This fixed amount of sugars increased with increasing concentration and increasing quantity of fed sugar solution, but decreased with progressing time after feeding. This fixed amount of sugars was independent of the metabolic rates of the bees and of the molarity and viscosity of the fed sugar solution.

As long as the bees did not exhaust their crop content, the haemolymph sugar titers were unaffected by the sugar surplus, by the time after feeding, by the concentration and by the viscosity of fed sugar solution. When bees were fed pure glucose (or fructose) solutions, unusually little fructose (or glucose) was found in the haemolymph, leading to lower total haemolymph sugar titers, while the trehalose titer remained unaffected.

In order to investigate the mechanisms underlying the regulation of the honeybee proventriculus, foraging bees were injected either with metabolisable (glucose, fructose, trehalose), or non-metabolisable sugars (sorbose). Bees reacted to injections of metabolisable sugars with reduced crop-emptying rates, but injection of non-metabolisable sugars had no influence on crop emptying. Therefore it is concluded that the proventriculus regulation is controlled by the concentration of metabolisable compounds in the haemolymph, and not by the haemolymph osmolarity. A period of 10min was enough to observe reduced crop emptying rates after injections. It is suggested that glucose and fructose have an effect on the proventriculus activity only via their transformation to trehalose. However, when the bees were already injected 5min after feeding, no response was detectable.

In addition it was investigated whether the overregulation is the result of feed-forward regulation for the imminent take-off and flight. In a first experiment, we investigated whether the bees release an extra amount of sugar solution very shortly before leaving for the hive. In a second experiment, it was tested whether the distance covered by the bees might have an influence on the surplus amount released prior to the take-off. In a third experiment, it was investigated if walking bees fail to release this extra amount of sugars, as they do not have to fly. Though we were not able to demonstrate that the overregulation is the result of feed-forward regulation for the imminent take-off and flight, it is conceivable that this phenomenon is a fixed reaction in foragers that can not be modulated.

To investigate whether regulated haemolymph sugar titers are also observed in honeybee foragers returning from natural food sources, their crop contents and haemolymph sugar titers

were investigated. While the quantity of the collected nectar was without influence on the haemolymph sugar titers, foragers showed increasing haemolymph sugar titers of glucose, fructose and sucrose with increasing sugar concentration of the carried nectar. In contrast no relationship between crop nectar concentrations and haemolymph trehalose titers was observed.

We are sure that the regulation of food passage from crop to midgut is controlled by the trehalose titer. However, under some conditions the balance between consumption and income is not numerically exact. This imprecision depends on the factors which have an impact on the foraging energetics of the bees but are independent of those without influence on the foraging energetics. Therefore we would assume that the proventriculus activity is modulated by the motivational state of the bees.

ZUSAMMENFASSUNG

Der Proventrikel reguliert den Nahrungstransport vom Kropf zum Mitteldarm. Weil die Verdauung erst im Mitteldarm stattfindet, kann alles, was im Kropf zurückbleibt, als Futter für Stockmitglieder und Brut verwandt werden. Die Aktivität des Proventrikels sollte daher bei Honigbienen so reguliert sein, dass die Sammlerin zwar mit ausreichend Energie versorgt wird, dass aber so viel Nektar wie möglich für den Bedarf der Kolonie im Kropf zurückbehalten wird. Da Honigbienen nur vernachlässigbare Mengen an Energiereserven besitzen, ist es unwahrscheinlich, dass diese am Regelsystem beteiligt sind.

Der Energieverbrauch der Sammlerinnen ist von ihrem Stoffwechsel abhängig, während die Energiezufuhr vom gesammelten Nektar abhängig ist. Daher ist anzunehmen, dass dies die beiden Hauptfaktoren sind, die sich auf die Proventrikelaktivität auswirken. Da die Hämolymphe einen stets aktuellen Einblick in den Ernährungszustand eines Insekts gewährt, kann man weiter annehmen, dass der die Proventrikelaktivität regulierende Faktor in der Hämolymphe zu finden ist.

Das Ziel der vorliegenden Doktorarbeit war es, die gegenseitige Beeinflussung von Aufnahme (Futterqualität und –quantität), Verbrauch (Stoffwechselrate) und „internal state“ Variablen (Hämolymphosmolarität und –zuckerspiegel) zu untersuchen und herauszufinden, welcher dieser Faktoren die Aktivität des Proventrikels bei der Honigbiene kontrolliert. Zu diesem Zweck wurden frei fliegende Sammlerinnen trainiert, kontrollierte Mengen verschiedener Zuckerlösungen zu sammeln. Direkt nach dem Füttern wurden die Stoffwechselraten über bestimmte Zeiten gemessen, danach wurden Kropfentleerungsraten und Hämolymphzuckerspiegel der jeweiligen Bienen gemessen.

Unter allen untersuchten Bedingungen waren sowohl die Zuckertransportraten durch den Proventrikel als auch die Hämolymphzuckerspiegel hauptsächlich von der Stoffwechselrate abhängig. Bei Bienen, die kontrollierte Mengen von 15-, 30- oder 50%-igen Saccharoselösungen gesammelt hatten, waren die Hämolymphtrehalose, -glucose und –fructosespiegel für Stoffwechselraten von 0 – 4,5 mlCO_2/h konstant. Bei höheren Stoffwechselraten sank die Trehalosekonzentration, während die von Glucose und Fructose stieg; eine Ausnahme stellten Bienen dar, denen 15%-ige Saccharoselösung gefüttert worden war. Da die Zuckerversorgung aus dem Kropf über den Proventrikel ausreichte, um auch die höchsten Stoffwechselraten zu ermöglichen, müssen die beobachteten Verläufe von einer Limitierung des Fettkörpers hinsichtlich der Trehalosesynthese herrühren. Die maximale Umwandlungsrate von Glucose zu Trehalose im Fettkörper wurde daher auf 92,4 µg Glucose/ Minute

berechnet. Allerdings war sowohl die Umwandlungsrate von Glucose zu Trehalose als auch die Zuckertransportrate vom Kropf in den Mitteldarm bei Bienen limitiert, die 15%-ige Saccharoselösungen gefüttert bekamen. Insgesamt führte das zu einem Absinken des Gesamt-Hämolymphzuckerspiegels bei Stoffwechselraten, die über 5 mlCO_2/h lagen. Hämolymphsaccharosespiegel waren generell niedrig, stiegen jedoch mit steigender Stoffwechselrate an; auch wenn nicht bei allen Bienen mit hohen Stoffwechselraten Saccharose nachgewiesen wurde.

Auch wenn die Sammlerinnen in der Lage waren ihre Zuckertransportrate genau an ihre Stoffwechselrate anzupassen, wurde unter bestimmten Bedingungen ein festgelegter Überschuss an Zuckern durch den Proventrikel transportiert. Dieser Überschuss an Zuckern vergrößerte sich mit zunehmender Konzentration und zunehmender Menge der gefütterten Zuckerlösung, verkleinerte sich aber mit fortschreitender Zeit nach dem Füttern. Er war unabhängig vom Stoffwechsel der Bienen und der Molarität und Viskosität der gefütterten Zuckerlösung.

So lange die Bienen ihren Kropfinhalt nicht aufgebraucht hatten, waren die Hämolymphzuckerspiegel von dem Überschuss an transportiertem Zucker, von der Zeitspanne zwischen Füttern und Hämolymphentnahme sowie der Konzentration der gefütterten Lösung und deren Viskosität unbeeinflusst. Wenn die Bienen allerdings reine Glucose- (oder Fructose-)lösungen gefüttert bekamen, wurde wesentlich weniger Fructose (oder Glucose) in der Hämolymphe gemessen, was zu niedrigeren Gesamt-Hämolymphzuckerspiegeln führte, während der Trehalosespiegel unbeeinflusst blieb.

Um den Mechanismus zu untersuchen, der der Proventrikelregulierung unterliegt, wurden Sammlerinnen mit entweder verdaubaren (Glucose, Fructose oder Trehalose) oder unverdaubaren Zuckern (Sorbose) injiziert. Die Bienen reagierten auf die Injektionen der verdaubaren Zucker mit einer Reduzierung der Kropfentleerungsrate, wohingegen die Injizierung nicht verdaubarer Zucker keinen Einfluss auf die Kropfentleerung hatte. Daraus wird geschlossen, dass die Proventrikelregulation von der Konzentration der verdaubaren Komponenten in der Hämolymphe kontrolliert wird und nicht von der Hämolymph-osmolarität. Eine Zeitspanne von 10min reichte aus, um nach der Injektion reduzierte Kropfentleerungsraten zu beobachten. Es wird angenommen, dass Glucose und Fructose nur über die Umwandlung zu Trehalose einen Einfluss auf die Proventrikelaktivität haben. Wenn allerdings die Injektionen bereits 5min nach der Futteraufnahme stattfanden, wirkte sich das nicht auf die Kropfentleerungsrate aus.

Weiterhin wurde untersucht, ob die Überregulation das Ergebnis einer „Vorschussregulation“ für den anstehenden Abflug und Flug ist. In einem ersten Experiment wurde untersucht, ob die Bienen diesen Überschuss erst direkt vor dem Abflug durch den Proventrikel lassen. In einem zweiten Experiment wurde untersucht, ob die Entfernung zwischen Stock und Futterquelle einen Einfluss auf die Menge des transportierten Zuckerüberschusses hat. In einem dritten Experiment wurde untersucht ob laufende Bienen auch einen Überschuss an Zuckern durch den Proventrikel leiten, obwohl sie nicht fliegen müssen. Auch wenn wir nicht nachweisen konnten, dass die Überregulation das Ergebnis einer Vorschussregulation für den anstehenden Abflug und Flug ist, ist es dennoch denkbar, dass dieses Phänomen eine festgelegte Reaktion der Sammlerinnen ist, die nicht moduliert werden kann.

Um zu untersuchen, ob man auch bei Sammlerinnen, die von natürlichen Futterquellen kommen, regulierte Hämolymphzuckerspiegel findet, wurden deren Kropfinhalte und Hämolymphzuckerspiegel bestimmt. Während die Menge des gesammelten Nektars keinen Einfluss auf die Hämolymphzuckerspiegel hatte, hatten Sammlerinnen höhere Glucose-, Fructose- und Saccharosehämolymphzuckerspiegel, wenn der Nektar im Kropf höher konzentriert war. Im Gegensatz dazu wurde keine Beziehung zwischen Nektarkonzentration und Trehalosespiegel gefunden.

Wir sind sicher, dass die Regulation des Futtertransports vom Kropf zum Mitteldarm über den Trehalosespiegel kontrolliert wird. Trotzdem ist die Bilanz zwischen Zuckertransportrate und Stoffwechsel nicht unter allen Bedingungen exakt ausgeglichen. Diese „Ungenauigkeit“ ist von denjenigen Faktoren abhängig, die einen Einfluss auf die Sammelenergetik der Sammlerinnen haben, aber unabhängig von den Faktoren, die keinen Einfluss auf die Sammelenergetik haben. Daher nehmen wir an, dass die Proventrikelaktivität über die Motivation der Bienen moduliert werden kann.

Acknowledgements

No one helped me...

Matt Ruff: *Sewer, Gas, Electric*. New York 1997.

No one finishes a doctoral thesis alone, and this one took longer than most; a lot of people helped me. Let me take you on a journey to meet them.

Descending the spiral staircase of the ivory tower we first stop gratefully and with a deep bow at the doors of Prof. Hölldobler, whose international reputation has enabled all of us to profit from talks given by the leading lights of the scientific community. Many thanks are also due to Prof. Tautz, whose prudent management of the funds of the Graduiertenkolleg has provided for some welcome financial subsidies. Another visit in theses upper regions is due to one of my predecessors in the field of this thesis, Prof. Núñez, who was one of the first to be interested in questions of proventriculus control and who was a valuable partner for discussions of the topic.

On the next floor below, we enter the newly painted office of PD Dr. Roces and thank him for the initial inspiration to work in this field; he was a major reason why I worked so hard and learned so much.

The middle regions of the tower, where the air is not quite as thin, are populated by a host of people of different qualifications and importance for this work – a general "Thank you" to all of them for just being there and the nice atmosphere. More specifically, I want to use this place to give hugs and kisses to my friend and colleague Simone Lohff, who shared both frustration and excitement over the years and who will remain in the treadmill for some more months. Nothing would have come out of this without the support and endurance of Eva Wirth, who analysed data and data and data over the years without complaints, and Annette Laudahn, who helped carrying out some of the experiments and also took care of the Endnote files – many thanks to you, as well.

The lower floors of the ivory tower are the dwelling place of all those helpful spirits without whose support nobody in the department would get anywhere. First of all, there's Mr Demmel, Keeper of the Beestation and Master of the Bees, who always thought straight when everybody else thought in circles. Then there is Mr Eisenmann, who provided the most valuable tools, probably because he works strictly according to the motto "A happy outcome is worth waiting for". And finally thanks are due to all the photographers, secretaries, cleaning ladies, librarians, etc., i.e. those who are in charge of all the minor worries that we don't have to care about.

When we go out onto the open field of normal life we meet a large gathering of people who always helped me not to get lost in this surreal place and who will hopefully provide me with enough ideas to spend my newly-won spare time.

TABELLARISCHER LEBENSLAUF

JASMINA BLATT

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BERUFLICHER WERDEGANG:

MAI 1990	Abitur am Immanual-Kant-Gymnasium in Neumünster
NOVEMBER 1990 – MAI 1996	Studium der Biologie an der Julius-Maximilians-Universität in Würzburg (Hauptfach Tierökologie, Nebenfächer Biochemie und Geobotanik) Diplomarbeit: Regulation des Proventrikels der Honigbiene (*Apis mellifera carnica*) in Abhängigkeit von der Futterqualität und der Aktivität der Bienen
MAI 1996 – MAI 2001	Promotionsstudium zum Thema „Haemolymph sugar homeostasis and the control of the proventriculus in the honeybee (*Apis mellifera carnica*)“ Doktoranden-Stipendium im Rahmen des DFG-Graduiertenkollegs „Grundlagen des Arthropodenverhaltens“ (01. Mai 1996 bis 15. August 1999) Wiedereinstiegsstipendium nach dem 3. Hochschulsonderprogramm (01.Juni 2000 bis 30. November 2000) Kooperation mit der Universität von Buenos Aires im Rahmen eines von DAAD und Fundación Antorchas geförderten deutsch-argentinischen PWA-Projekts (Auslandsaufenthalt in Buenos Aires von 1. Februar 1998 bis 30. April 1998) Betreuung diverser tierphysiologischer Praktika
01. MÄRZ 1999	Geburt meiner Tochter Moira Blatt

Würzburg, den 28.05.2001

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Jasmina Blatt

PUBLICATIONS

full papers:

1 Roces, F. Blatt, J. 1999. Haemolymph sugar and the control of the proventriculus in the honeybee *Apis mellifera.* Journal of Insect Physiology 45, 221-229

2 Blatt, J. Roces, F. 2001. Haemolymph sugar levels in foraging honeybees (*Apis mellifera carnica*): dependence on metabolic rate and *in vivo* measurements of maximal rates of trehalose synthesis. Journal of Experimental Biology, in press

3 Blatt, J. Roces, F. 2001. The control of the proventriculus in the honeybee (*Apis mellifera carnica* L.) I. A dynamic process influenced by food quality and quantity? Journal of Insect Physiology, submitted

4 Blatt, J. Roces, F. 2001. The control of the proventriculus in the honeybee (*Apis mellifera carnica* L.) II. Feedback mechanisms and functional questions.? Journal of Insect Physiology, submitted

in prep:

5 Blatt, J. Roces, F. Haemolymph sugar levels of honeybees (*Apis mellifera carnica* L.) feeding on natural food sources.

6 Blatt, J. Roces, F. The occurrence of sucrose in the honeybee haemolymph (*Apis mellifera carnica* L.).

7 Roces, F. Blatt, J. Differences in the proventriculus control between the two honeybee races *apis mellifera carnica* and *apis mellifera ligustica*

conference article:

8 XX INTERNATIONAL CONGRESS OF ENTOMOLOGY
Firenze, Italy, 1996 ”Haemolymph sugar and the control of the proventriculus in the honeybee *Apis mellifera*
J. Overath, F. Roces, Theodor-Boveri-Institut, Lehrstuhl für Verhaltensphysiologie und Soziobiologie der Universität, Würzburg, Germany

9 16TE TAGUNG DER DEUTSCHSPRACHIGEN SEKTION DER IUSSI
Graz, Österreich, 1997 ”Regelung des Proventrikels bei der Honigbiene *Apis mellifera* in Abhängigkeit von der Aktivität und dem Hämolymphzuckerspiegel der Bienen”
Blatt J., Roces F, Theodor-Boveri-Institut, Lehrstuhl für Verhaltensphysiologie und Soziobiologie der Universität Würzburg, 97074 Würzburg, Germany

10 INTERNATIONAL SYMPOSIUM ON ANIMAL PHYSIOLOGY
Bogensee, Deutschland, 1998 ”The regulation of the proventriculus activity in the honeybee (*Apis mellifera carnica*) depends on the metabolism.”
Blatt J., Roces F, Theodor-Boveri-Institut, Lehrstuhl für Verhaltensphysiologie und Soziobiologie der Universität Würzburg, 97074 Würzburg, Germany

11 6TH EUROPEAN CONGRESS OF ENTOMOLOGY
Budweiss, Tschechoslowakai, 1998 ”Sugar levels in the honeybee haemolymph (*Apis mellifera carnica*) depends on the ingested sugar solution and on metabolism.”
Blatt J., Roces F, Theodor-Boveri-Institut, Lehrstuhl für Verhaltensphysiologie und Soziobiologie der Universität Würzburg, 97074 Würzburg, Germany

Erklärung

Hiermit erkläre ich, die vorliegende Arbeit in allen Teilen selbständig und nur mit den angegebenen Hilfsmitteln und Quellen angefertigt zu haben.
Ich erkläre weiterhin, dass diese Dissertation weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen hat.
Zudem erkläre ich hiermit, früher weder akademische Grade erworben zu haben, noch habe ich versucht solche zu erlangen.

Würzburg, den 28.05.2001