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Measuring the sugar consumption of larvae in bumblebee micro-colonies: a promising new method for tracking food economics in bees

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Abstract – The consumption of sugar is an important part of the energy intake of social insect. Its monitoring provides information regarding the costs and efficiency of energy flow in a colony. This study aims at tracking the sugar flow from a sugar source to artificial bumblebee micro-colonies and at quantifying the amount of sugar consumed by the larvae. We developed a new method of sugar tracking that utilises an inert lanthanide complex (GdDOTA) dissolved in an aqueous sugar solution. The delayed defecation of bee larvae enabled the collection of all faeces from a cocoon. The amount of digested sugar corresponded to the amount of the lanthanide in the faeces, which was quantified using inductively coupled plasma spectrometric techniques. We highlight the possibility of the novel developed method to be extended for tracking the energy flow within a colony using up to 15 different metal markers without the necessity of killing individuals.

marking technique / lanthanide / carbohydrate / insect / ICP-OES

1. INTRODUCTION

Social bees, like every living organism, require energy for each of life activities, growth, reproduction and all other life processes. "Like machines, bumblebees must be supplied with fuel.

The only fuel they can use is sugar. As in the internal-combustion engine, the utilization of a given amount of fuel by a bee requires the intake of a given amount of oxygen. Carbon dioxide, water, and heat are produced as by-products. The metabolic rate, or rate of fuel utilization, is a measure of an organism's rate of living. It can theoretically be measured either by what goes into the system or by what comes out" (Heinrich 2004). Although Heinrich was referring to adult bumblebees, and especially the expenditures of flying workers, the same relationship applies to all of the physical activities and physiological processes in the adults and in the developing larvae.

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Energy flows and costs are not yet fully understood in social bees. Furthermore, Heinrich (2004) noted that bumblebees require a lot of energy to keep their colonies growing, but relatively little is known about the resources, their allocations and amounts because the bees generally live from hand to mouth. Obviously, the quality of the resources is very important (Goulson 2003). A bee's diet comprises proteins from pollen and carbohydrates from nectar. Diets from different types of pollen contain between 2.5 and 61 % total protein (Roulston et al. 2000), and this variation can have a large influence on bee brood size (Roulston and Cane 2002). However, it was shown that the quality of nectar (sugar concentration) does not have a large influence on the brood size (Quezada-Euán et al. 2011). Although carbohydrates are always included in the larval provisions, their function and importance regarding larval development are known only marginally. On one hand, food is known to be important in the regulation of phenotypic expression of caste-specific larval development in some bumblebees (Pendrel and Plowright 1981), but on the other hand, food composition (sugar/protein ratio) can be stationary during the development of the larvae and may not differ among the castes within a bumblebee colony (Pereboom 2000). However, the latter is not true for the honeybee, in which the differences in food supplies among castes are firmly established (Tautz 2009).

Complexity of the nutritional flow in a bee colony indicates a need for a suitable experimental approach of food distribution monitoring throughout a colony. The use of markers for tracking a substance of interest is a widely applied approach, and it is utilised in such different fields, as wastewater monitoring in civil engineering (Rieckermann et al. 2007) or for imaging of processes in living cells (Nienhaus 2008). Experiments involving marker tracking have already been performed with bees. The nectar transmission within a honeybee community has been studied using radioactive phosphorus (Nixon and Ribbands 1952) or a protein marker (DeGrandi-Hoffman and Hagler 2000). However, working with radioactivity brings demanding safety issues and marking with proteins does not allow the data to be quantified. All marker

types, which were used previously in insects, have been reviewed by Hagler and Jackson (2001).

Inductively coupled plasma (ICP) techniques are analytical methods allowing for the precise quantification of elements, typically in sub-ppm concentrations using optical emission detection (OES), or even in sub-ppb concentrations using mass spectrometry detection (ICP-MS). The lanthanides is a group of 15 elements with very similar chemical properties exhibiting moderate to high sensitivity to ICP-OES. Yttrium and Scandium exhibit similar properties to lanthanides, and all 17 elements can be used in a manner described in this article. The lanthanide–ICP marker–detection pair was proposed in the 1990s (Allison and Baugh 1994), and the concept has already been used in various fields, including proteomics and protein analysis (Tholey and Schaumlöffel 2010), tumour-type determination in histological preparation (Giesen et al. 2011), and other fields (e.g. Řehoř et al. 2011). The use of lanthanide markers in living systems appears to be questionable considering their high toxicity. The use of advanced multidentate cyclic chelators (such as DOTA-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) gives rise to lanthanide complexes of such a high thermodynamic stability ($\log\beta > 20$) as well as kinetic stability that no detectable release of free toxic lanthanide from the chelate occurs (Merbach and Tóth 2001). The nontoxicity of these chelates is demonstrated by their use in human medicine—trivalent gadolinium DOTA complex (GdDOTA) is used as a contrast agent in magnetic resonance imaging, in which one administration represents a dose of several grams of GdDOTA. The complex does not enter the cells and is rapidly cleared from the body in an unchanged form (Merbach and Tóth 2001); half-time for a rat is 18 min (Bousquet et al. 1988).

This paper aims at tracking the sugar distribution in artificial bumblebee nests, and especially at quantification of the sugar amount consumed by the larvae. Hence, practical application of our newly developed method is demonstrated. Our new approach uses a nontoxic complex of rare earth element as a marker that is dissolved in an aqueous sugar solution. The amount of sugar consumed is equivalent to the amount of lanthanide in the faeces, which can be determined exactly using ICP-OES.

Thus, we have detected simple paths of sugar flow in the colonies and have quantified the amount of sugar consumed by the larvae.

2. MATERIAL AND METHODS

2.1. Experimental bumblebee micro-colonies

We used artificially bred *Bombus terrestris* (Agricultural Research, Ltd. Troubsko) for our experiments. The species belongs to the pollen-storer group of bumblebees. This means that the workers feed the larvae through holes made in a wax cover. Like other bees, *B. terrestris* larvae store the faeces from all consumed food in the midgut until the lumina of the posterior part of the intestines disappear and the faeces come out (Alford 1975). This happens during the final part of development of the fourth larval instar, usually around the time of cocoon spinning (Michener 2007). All faeces are voided and smeared over the cocoon's inner surface (Michener 1974, Kearns and Thomson 2001).

We prepared artificial micro-colonies (Regali and Rasmont 1995) with three workers. Callow workers from the same nest and of a comparable size were selected to join the colony (individuals weighed and compared). They were placed into plastic containers (PETRA plast Ltd) with dimensions 14×19×17 cm. Two sets with 20 colonies each were prepared. The sugar solution with chemical tracer (GdDOTA) was placed in the colonies M1-M20, while the control colonies C1-C20 were supplied with the Gd free sugar solution. Preparation of the sugar solution is described below, while the preparation procedure of GdDOTA complex is given in Supplementary information (see Method SI1). All control colonies were checked for the risk of contamination from Gd-marked sugar solution. This type of experimental micro-colony can produce only male broods (Regali and Rasmont 1995).

2.2. Preparation of the sugar solution with GdDOTA

The sugar solution for the bumblebee colonies was prepared once at the beginning of the experiment. It was a mixture of saccharose (1,000 g, white beet sugar, local producer) and fructose (460 g, Fructopur®, Natura)

dissolved in water (1,200 g). Its density at room temperature was 1,214 g/L. A solution of GdDOTA was prepared according to previously published procedure (Bousquet et al. 1988; Aime et al. 1997); preparation details are described in [Supplementary information](#). Ten milliliters of GdDOTA solution (2.6 mmol/L) was added to 1 L of the sugar solution. The resulting concentration of Gd in the feeding sugar mixture was 403 mg/L (concentration verified by ICP-OES), the concentration of saccharose was 452 g/L and the concentration of fructose was 207 g/L (conversion coefficient, 1.00 mg Gd corresponds to 1.63 g of sugar). The content of Gd in sugar solution was estimated using ICP techniques. The sample was too viscous for direct analysis, and the removal of high organic content was needed (Method SI2). The sugar solution for the control colonies was prepared analogously, but with an equivalent amount of deionized water instead of the GdDOTA solution.

2.3. Nectar and pollen feeders

Only the sugar solution and pollen were added to the feeders to replenish them. A known volume of the sugar solution was added to the 10-mL plastic feeder (Tachovské SPV Ltd) with a syringe. Honeybee pollen pellets (a mixture of pollen with dominance of Brassicaceae, Rosaceae and Papaver from a local beekeeper) were weighed before being added to the pollen feeder. All of the colonies were supplied with sugar and pollen according to their needs, and the consumption of both nutrients was monitored during the course of the experiment. Supplies were added after consumption of major part of the previous.

2.4. The course of experiment and its termination

All of the colonies were observed regularly (6 days per week), their development was checked and their progress was noted (pollen and nectar consumption, construction of brood cells, cocoon development and emergence of offspring). Micro-colony keeping was successfully terminated after the pupation of the last larva of the first brood generation. When one of the workers died before the pupation of the last larva of the first brood generation, the experiment was terminated prematurely, and the results from this unsuccessful

colony were not analysed. When the colonies were successfully terminated, all of the workers were frozen, and the cocoons of the first generation were separated and reared independently to compare the mortality of the Gd-marked broods to the control broods. The cocoons from all of the colonies were carefully handled; however, separation of the cocoons from one clutch was complicated, and many cocoons were partly compressed. The separated cocoons containing the living pupae were stored in plastic Ziplock bags until the bees' emergence. The following basic characteristics and course of development were noted for comparison between the Gd-marked colonies and the unmarked colonies: the sum of mass of the three workers, the number of pupated larvae, the number of offspring from the first generation, the sum of the offspring mass, the average offspring mass, the number of days from the first egg cell to the first cocoon, the number of days from the beginning of the experiment to the first egg cell, the sum of the weight of the incoming pollen and the sum of the volume of the incoming sugar solution.

2.5. Body mass analysis of the bumblebee brood

All of the individuals from the experimental bumblebee offspring ($N=123$) were stored in the freezer until body mass was determined. The analysis includes emerged and dead (not emerged) adults and black pupae. Individuals that failed to emerge or that died were removed from the cocoon, and the cocoons were subsequently analysed for Gd content in the same way as the cocoons of emerged individuals (see below). The individuals were dried in an oven at 50 °C for 5 days. The dried bodies were weighed, placed back into the heater for another 2 days and weighed again. When the weight between the two subsequent measurements differed by more than 1 mg, the individual was placed back into the heater for another 2 days. This process was repeated until a stable mass was obtained.

2.6. Gd content analysis in unused sugar provisions from nectar feeders

To determine the sugar amount that did not enter the colony, the sugar feeders were rinsed after the end

of the experiment. The following procedure was applied:

The sugar feeder and its contents were placed in hot water (100 mL) and rinsed for 1 min while stirring.

The rinse was repeated with cold water (100 mL) after decanting the solution.

Both rinsing solutions obtained in previous steps were mixed together and frozen until it was processed for the subsequent determination of Gd content.

The previously frozen solutions were defrosted and thoroughly mixed.

Ten milliliters of solution was filtered into a clean vial through a 0.45- μ m syringe filter.

The aliquot of 5 mL of each sample was used for further treatment. Before measurement, 0.22 mL of 65 % HNO_3 (suprapur grade, Fisher Scientific) was added to the aliquot sample to get 2 % final HNO_3 concentration.

2.7. Gd content analysis in the bee colony waste

In order to establish the total loss of Gd marker during experiment, we analysed the content from the whole colony cage (waste). This waste includes the cage itself, the wax remnants and the honey pots that workers made from wax and the pollen feeder, including unused pollen. It does not include the unused sugar solution, the worker bumblebees and the cocoons with pupae/offspring (each of them was analysed separately). The following steps were performed:

The waste was rinsed with hot water (150 mL) using 1 mL of detergent for 3 min, and then, it was mixed and poured into a glass vial.

The rinsing procedure was repeated twice more in cold water (twice 50 mL).

In total 250 mL of the solution from the waste rinsing steps was frozen until processed for the subsequent analysis of Gd content.

The samples were defrosted and thoroughly mixed.

Ten milliliters of solution was filtered into a clean vial through a 0.45- μ m syringe filter.

The aliquot of 5 mL of each sample was used for further treatment. Before measurement, 0.11 mL of 65 % HNO_3 was added to the sample to get 2 % final HNO_3 concentration.

2.8. Gd content analysis in the cocoons

For the quantification of sugar consumed by larvae, we separately analysed each bumblebee cocoon according to following procedure:

Bumblebee cocoon was placed into a glass vial. Distilled water (8 mL) and the detergent Triton X-100 (0.30 mL, Sigma-Aldrich) were added and the mixture was stirred.

The vials were heated to the point of boiling. Subsequently, the cocoon was broken into pieces using a metal spatula; the mixture was thoroughly mixed and then boiled for 5 min.

HNO_3 (0.31 g of 65 %, Suprapur grade, Fisher Scientific) was added to a new weighed polypropylene vial.

The cold Triton extract was filtered through a PTFE syringe filter (pore size 0.45 μm , Fisher Scientific) to the vial with HNO_3 . HNO_3 was used for the stabilisation of solutions for long-term storage.

The empty vial from the sample was washed carefully with water (~2 mL) and added to the rest of sample through the same filter.

The sample was filled with distilled water to a final mass of 10.00 g (final HNO_3 concentration was 2 %).

2.9. Test of Gd content loss during rinsing of the cocoons

In order to establish the rate of recovery of the GdDOTA from cocoons, we proceeded as follows. Empty cocoons from artificial colonies were used for this experiment. The first group of empty cocoons ($N=15$) was labelled with Gd DOTA solution (1.18 μL , 0.31 M; 5.56 μg Gd was added inside each cocoon). The cocoons were kept in the freezer for 2 days before the rinsing process. The rinsing process and preparation of the samples for analysis was carried out following the protocol for cocoon processing described above. The

second (control) group of cocoons ($N=15$) was processed in the same manner, but the GdDOTA solution was added to the extract solution after cocoon rinsing and filtering.

2.10. Gd content analysis on the surface and inside bumblebee brood and workers

The following experiment was performed to see whether bumblebee brood ($N=11$) and workers ($N=9$) contain lanthanide tracer inside or on the surface of their bodies. Firstly, the content of tracer on the surface of the body of each individual (brood or worker) was estimated:

Each individual was put to preheated (to 80 °C) mixture of distilled water (8 mL) with the detergent Triton X-100 (0.30 mL).

Solution with bumblebee was gently stirred at 80 °C for 15 min.

HNO_3 (0.31 g of 65 %) was added to a new weighed polypropylene vial.

The cold Triton extract was filtered through a PTFE syringe filter (pore size 0.45 μm , Fisher Scientific) to the vial with HNO_3 .

The empty vial from the sample was carefully washed with water (~2 mL) and added to the rest of the sample through the same filter.

The sample was filled with distilled water to a final mass of 10.00 g (final HNO_3 concentration was 2 %).

Then, the content of tracer inside the bodies of the same individuals was estimated as follows:

Bumblebee was dried completely in the heater.

Afterwards, it was placed in a PTFE cup.

The body was broken to small pieces by a glass spatula.

HNO_3 (~2 mL) was added and the mixture was heated to 150 °C until completely dry.

After cooling, 0.31 g HNO_3 was added to the solution and dried again at 60 °C. The process was repeated if necessary, until the HNO_3 solution was clear.

Subsequently, 0.31 g HNO_3 was added to dry residue, the mixture was stirred and prepared solution was transferred to a new vial.

PTFE cup was washed twice by distilled water and the solution formed was mixed with HNO_3 solution.

The sample was filled with distilled water to a final mass of 10.00 g (final HNO_3 concentration was 2 %).

2.11. Instrumentation and calibration for the analysis of Gd quantity

The ICP-OES (inductively coupled plasma optical emission spectroscopy) measurements were performed using the SPECTRO ARCOS optical emission spectrometer (SPECTRO Analytical Instruments, Kleve, Germany) with a radial plasma observation. The SPECTRO ARCOS features a Paschen-Runge spectrometer mount; the wavelength range between 130 and 770 nm can be simultaneously analysed. An air-cooled ICP-generator, based on a free-running 27.12 MHz system, was installed. For sample introduction, a Cross-Flow nebuliser with a Scott-type double pass spray chamber was used.

For the calibration, commercially available single element standards of Gadolinium and Yttrium (nitrate) solutions (Analytika) were used. In all cases, 5.00 mg/L Y was used as an internal standard. The Y II 324.228 nm, Y II 349.609 nm, Y II 360.073 nm and Y II 371.030 nm lines were used for internal standardisation. All of the calibration measurements were performed in 2.0 % (w/w) HNO_3 (prepared from 67 %, Suprapur grade, Analytika) as a basic matrix. All of the chemicals used were of Suprapur grade.

Samples were homogenized for 5 min in an ultrasonic bath. Then, 5-mL aliquots of each prepared sample were taken. If not already performed, each sample was spiked with 25.0 μL of a Y standard solution (1.00 mg/mL) and mixed. Each sample was measured in 2 % HNO_3 under specific ICP-OES measurement conditions (Method SI3).

2.12. Processing the results from ICP-OES

The amounts of Gd (m) in separate samples (unused sugar solution in feeder—F, cocoon—C, and waste—W) were calculated from the volume (V) of the sample (F—200 mL, C—10 mL, W—250 mL) and the measured concentration (c) from ICP-OES: $m_{(\text{F,C,W})} = V_{(\text{F,C,W})} \times c_{(\text{F,C,W})}$. The total weight of the Gd (m_{tot}) from the sugar solution loaded into a single colony was calculated as the sum of the results of all measurements

belonging to that colony: $m_{\text{tot}} = m_{\text{F}} + \sum m_{\text{C}} + m_{\text{W}}$. The amount of sugar consumed by the larvae (m_{S}) was calculated from the amount of Gd found in the cocoons. Regarding sugar quantification, the Gd concentration was corrected by the approximation of Gd loss during the rinsing of the cocoons, which was tested in our study and multiplied by a conversion coefficient between the Gd and the sugar: $m_{\text{S}} = m_{\text{C}} \times 1.097 \times 1.63$.

2.13. Statistical analyses

The data from both the control and the experimental groups were tested for normality (Shapiro–Wilk test) and homogeneity of variances (F test, and Bartlett, Hartley, Cochran and Levene tests). The groups were compared mutually using a t test. Welch's t test was applied if some of the homogeneity tests implied that there was unequal variance among groups. A Mann–Whitney U test was computed for non-normally distributed or discrete data. There were no multiple testing problems because none of the control-comparing tests was significant, and the aim of this study was to demonstrate that there is no difference between the control and the experimental groups. The relationships between the quantitative variables were assessed using a standard linear regression (when dependent and independent variables could be meaningfully distinguished) or a Pearson correlation (when dependent and independent variables could not be distinguished). All of the statistical analyses were performed using Statistica 10 software (StatSoft Inc. 2011).

3. RESULTS

3.1. Development of marked and control bumblebee micro-colonies

Twenty-two micro-colonies completed successfully their development to pupation of all broods from the first generation. Eleven micro-colonies were from the experimental Gd-marked set, and the same number was from the control set. Two control colonies were excluded from comparison of controls with marked colonies because of contamination from Gd-marked sugar solution during the course of experiment. Apart from the described cases of contamination, the Gd background level in all

of the other control samples was three orders of magnitude smaller than in our positive samples.

We found that the consumption of pollen and nectar varied among colonies, but consumption of both nutrients was significantly correlated (Pearson $r=0.756$, $P<0.0001$) within all of the successfully terminated micro-colonies.

Separated cocoons with brood from terminated micro-colonies were kept in plastic bags to emerge. The stored cocoons suffered from fungal infections, or emerging males were often unable to bite their way out of the cocoon. These difficulties are most likely responsible for the low number of emerged brood individuals from both the control and the marked colonies. Thirty-three and 23 (of 123 in total) individuals were removed from the cocoons in the control and the experimental colony groups, respectively. All of these individuals were at least pharate imagoes in the pupal cuticle, except for three cases, in which we could not determine the development stage because these specimens were dead for a longer time and decomposed in cocoon. Subsequently, all of the un-emerged offspring, which reach adult stage (sometimes still in a pupal cuticle), were analysed like the emerged ones. We compared statistically the summary mass of all three workers, the number of cocoons, the number of emerged offspring, the average offspring mass and the sum of the offspring mass, the length of development from the first egg cell to the cocoon, the time from the beginning of the experiment to the first egg cell and the amount of consumed pollen and nectar per colony (Table I) between the Gd-treated and the control colonies. We found no significant differences ($P>0.05$) in any of these characteristics (Table SI1).

3.2. Test of Gd content loss during the rinsing of the cocoons

Gd quantification of the cocoons from the marked micro-colonies is a key method in our experiment. It is obvious that there can be losses during the manipulation of samples and during the rinsing of Gd from the cocoons. We determined these losses in a test with two groups of empty

cocoons. The test group of cocoons was labelled with known amount of GdDOTA (label was injected inside the cocoon), and the cocoons were treated using a standard rinsing procedure. In the control group, the label was added to the rinsed solution from extraction of label-free cocoons in order to have no losses of Gd content in final sample. In the tested rinsed samples ($n=15$; Av. 5.293 mg/L Gd; StDev=0.179), the concentration of Gd was 8.82 % lower than the concentration of Gd in the controls ($n=15$; Av. 5.805 mg/L Gd; StDev=0.246). This difference between the rinsed samples and the control samples is significant (T test, $t=6.298$, $P<0.00001$).

3.3. Gd content analysis inside bumblebee workers and brood and on their body surface

As it was theoretically expected, no or insignificant traces of Gd (0.1 μg on average) were found inside the bodies of bumblebee brood, and thus, no food remains in intestines of freshly emerged bumblebee individuals. Only slightly higher content of Gd was found on the body surface of brood (0.7 μg on average); thus, minor contamination from faeces occurred. This means less than 1 % of total sugar content was found in a single cocoon on average. Workers from terminated colonies contained up to 51.6 μg of Gd (=84 mg of sugar) (30.1 μg of Gd on average) and 17.5 μg of Gd (4.8 μg on average) on the body surface. The results are summarized in Table SI2.

3.4. Recovery of Gd entering the experimental colony

The Gd element entered the micro-colony from a sugar feeder, which was the only source of sugar for the three workers in the colony. The analyses of the remaining sugar solution, the cocoons and the waste from the colony cage showed that Gd was distributed in all of the parts of each of the colonies at the end of the experiment (Table II). We compared the sum of the Gd mass measured by ICP-OES in the remaining sugar solution, all of the cocoons and the waste from the experimental colony cage

Table I. Summary of basic statistics and a comparison of the control (C3-20) and the Gd-marked (M1-20) bumblebee colonies.

Colony	Sum of m of three workers (mg)	No. of cocoons	No. of emerged offspring	Sum of offspring mass (mg)	Average offspring mass (mg)	Days from first egg cell to cocoon	Days from beginning to egg cell	Sum of pollen (mg)	Sum of sugar solution (mL)
C3	664	6	0	278	46	17	7	4,154	32
C4	485	7	4	409	58	15	7	4,379	28
C6	317	6	1	305	51	16	9	3,994	26
C9	633	8	4	388	48	22	8	5,600	35
C10	595	9	1	478	53	32	7	6,607	41
C16	594	12	8	468	39	18	7	6,441	30
C17	627	2	0	115	57	19	8	4,035	27
C19	583	4	3	167	63	13	8	3,608	26
C20	571	5	3	314	42	16	13	3,608	27
M1	641	9	5	388	48	17	7	4,857	25
M6	460	7	6	292	42	15	9	4,587	26
M7	739	4	3	199	50	20	7	3,836	29
M8	521	2	1	112	56	16	8	3,222	26
M11	476	10	7	423	42	16	9	5,429	30
M12	432	4	1	184	46	19	9	4,441	22
M13	481	7	2	388	55	17	11	4,693	32
M16	626	5	2	273	55	18	7	3,833	24
M17	662	3	2	171	57	16	13	4,535	28
M18	670	8	5	363	45	21	9	4,275	34
M20	527	5	4	195	49	26	8	5,793	35
C vs. M	NS	NS	NS	NS	NS	NS	NS	NS	NS

Measuring of the weight was not possible in one M1 and one M20 brood individual because of fungal infection and these are not included to offspring mass values.

Comparison of the control and the experimental group regarding colony development variables represents bottom row C vs. M. The results of both a *t* test and a Mann–Whitney *U* test are in supporting information Table S11

NS not significant

with the theoretically expected Gd mass loaded into the colony with the sugar. We found that the Gd mass recovery from our experiment is significantly lower than the expected Gd mass (pair *t* test, $t=5.654$, $P<0.0005$), but a positive correlation between the measured and estimated values is obvious (linear regression without intercept, $r^2=0.478$, $P=0.013$, Figure 1). On average, 77 % of the total amount of Gd added to the individual colony was recovered. The variability in the results from the different

colonies is relatively large (48–96 % of returned Gd). The results from the individual colonies are summarised in Table II.

3.5. Consumption of sugar by larvae during their development

The larval consumption of sugar is proportional to the Gd content detected in their cocoons. For the sugar quantification, we always used a correction for the approximated 8.82 % loss

Table II. Summary of the Gd content in separately analysed parts of the experiment, and the percentage of Gd return counted from the total input sugar solution volume.

Colony	Rest of sugar solution (mg)	Rinse of cage (mg)	Sum of cocoon rinse (mg)	Total Gd (mg)	Expected Gd (mg)	Gd return (%)
M1	1.779	2.520	0.552	4.852	10.081	48.13
M6	2.257	5.739	0.542	8.539	10.485	81.44
M7	2.626	6.867	0.340	9.832	11.694	84.07
M8	2.114	5.858	0.227	8.200	10.485	78.21
M11	1.643	6.492	0.623	8.758	12.098	72.40
M12	1.430	4.757	0.381	6.568	8.872	74.03
M13	1.742	7.483	0.729	9.954	12.904	77.14
M16	2.427	6.634	0.257	9.317	9.678	96.27
M17	1.404	7.879	0.228	9.511	11.291	84.23
M18	1.349	6.862	0.494	8.705	13.711	63.49
M20	3.114	9.587	0.336	13.037	14.114	92.37
Av						77.43

during the Gd rinse from the cocoons; thus, we detected 128 mg of sugar in one cocoon of one individual male bumblebee on average. There is little variability among colonies in the sugar amount per brood individual (Table III). The amount of sugar content in the cocoon depends positively on the weight of an individual (linear regression, $r^2=0.291$, $P=0.0001$, Figure 2). This relationship shows that a change of 10 mg in brood body mass during development equals a consumption of 29 mg of sugar in our experiment.

4. DISCUSSION

In presented work, we tracked sugar distribution with a labelled sugar solution in the artificial bumblebee micro-colonies and quantified the amount of sugar consumed by the bumblebee larvae during their entire development. Sugar transmission has been studied using radioactive tracer (Nixon and Ribbands 1952) or technique that utilises proteins and immunological detection (DeGrandi-Hoffman and Hagler 2000); however,

Figure 1. The plot shows the relationship between the total Gd content in a colony and the amount of administered sugar. The plot displays amounts of Gd in the original sugar solution, calculated using the known Gd concentration (dashed line), experimental data from our colonies (black squares) and their regression (solid line).

Table III. Consumption of sugar by the larvae of bumblebees in our Gd-marked colonies.

Colony	Clutch size	Av. mass of brood (mg)	All sugar in clutch (mg)	Av. sugar per individual (mg)
M1	9	48	990	110
M6	7	42	972	127
M7	4	50	609	139
M8	2	56	407	186
M11	10	42	1,117	102
M12	4	46	683	156
M13	7	55	1,307	170
M16	5	55	460	84
M17	3	57	409	124
M18	8	45	886	101
M20	5	49	602	110
Av		50	768	128
StDev		5	291	30

The amount of sugar is counted from the Gd content and includes an approximated 8.82 % Gd loss during the rinsing of the cocoons

the latter does not allow quantification. Contrary to these techniques used in honeybee colonies, our novel technique of food source marking in bee colonies is nonradioactive, safe and easy to handle and allows the quantification of a marked target.

When any labelling technique is used in experiment with living organisms, the presence of the label cannot influence the results of the study. Thus, we tested survival of our bumblebee colonies labelled with GdDOTA as well as

number of offspring, development time and other important parameters related to colony viability and progress. Marked colonies did not differ significantly from control colonies in any of such parameters, indicating that label was inert in our experimental setup. The nontoxicity of our label was reported previously for vertebrates including humans (Merbach and Tóth 2001). Hence, this study confirmed the nontoxicity also for insects. Furthermore, no

Figure 2. The amount of sugar consumed by individual larvae, based on the analysis of the Gd mass found in their cocoons, increases linearly with the dry weight of the offspring at the adult stage; regression line $y=2.8722x+3.509$.

accumulation in brood bodies was observed, which indicates total clearance (also previously reported for vertebrates—Bousquet et al. 1988).

Until now, it was not attempted to detect the necessary amount of sugar to feed one larva in the progressively provisioning bees, such as honeybees and bumblebees, because they feed the larvae continuously through their entire larval development. Only Nixon and Ribbands (1952) quantified disposable amount transferred from a worker bee to two individual larvae using radioactivity. On the other hand, in the case of mass provisioning stingless bees, it was relatively easy to determine the amounts of pollen and nectar and their proportions in individual nourishment because these species lay their eggs on the completed provisions in the cell (Quezada-Euán et al. 2011). However, the question is how much of the supplies they really eat.

Using our methodology, we were able to quantify the amount of sugar (128 mg, on average) consumed by male larvae during their development. We detected that the average male bumblebee in our experiment had to consume 29 mg of sugar per every 10 mg of its body mass during development. Furthermore, we demonstrated that the colony consumption of sugar and the colony consumption of pollen are significantly correlated variables. These relationships are definitely case-specific for our micro-colony settings and may vary in different contexts. The data are very likely affected by the fact that we fed our colonies *ad libitum*, which enabled a steady rate of feeding on both sugar and pollen throughout the course of experiment. However, the correlation between the consumption of sugar and the consumption of pollen is not commonplace, as suggested by field studies on mass provisioning stingless bees (Quezada-Euán et al. 2011). It indicates that the proportion of sugar and pollen consumption can vary in time according to both the pollen and the nectar quality which are naturally available for foragers. It is not surprising that the same study also did not show any effect of the sugar amount on brood growth but that it did show a strong dependence of this variable on the amount of pollen (amount of protein). The results of that study indicate that stingless bees add more sugar to

the provision, most likely as food compensation, when a limited amount of pollen supplies is accumulated.

Not only nectar but also pollen can be marked and tracked with lanthanides. The only requirement is that the pollen must be mixed with the lanthanides in a known ratio. This can be performed according to the methods used by Cnaani et al. (2002). The marked pollen can also be tracked together with the nectar in future experiments, when each of them is marked by a different lanthanide. Thus, the utility of our new method for future experiments on bees in which the quantity and quality of food has been manipulated is evident.

The total Gd recovery from our micro-colony after the experiment averages 77 % of the administered dose. Despite this loss in the total Gd recovery, the obtained data follow logical and expected trends, indicating that this systematic Gd loss is not affecting the results by random biases. Moreover, the rinsing of Gd from the cocoons, where we direct most of our attention, produces only 8.82 % of Gd loss on average, with little variation. The total recovery may be significantly increased by using harsh rinsing methods (e.g. mineralisation in HNO_3), but our mild extraction method, using only water and detergent, is fast, convenient and gives sufficiently precise data.

Our technique is perfectly suitable for bee and wasp models, in which the offspring develop in separate cells or cocoons and defecate at the end of the development. This behaviour allows the collection of all of the faeces from an individual. Our marking technique could be optimised for use in complex studies of social bee colonies, in which we could observe colony energetics without the necessity of killing any individual. Furthermore, it would allow the observation of bee colonies of subsequent generations and the comparison of energetics between new and ancestral colonies. Hence, direct tests of heredity characteristics that presently cannot be observed directly would become possible.

We should notify that users of this method can use up to 14 lanthanides (extremely rare and radioactive Promethium is excluded), as well as Scandium, Yttrium and also any combination of

lanthanides can be applied. For the case of field studies, it is necessary to note that lanthanide concentrations in the Earth's crust vary from 68 ppm for Cer down to 0.5 ppm for Thulium. Lanthanide deposits are diffuse, and therefore, samples from the soil can be contaminated by naturally occurring ions. Fortunately, lanthanides in nature are usually in the form of highly insoluble salts (phosphates), and the contamination during the rinsing process is therefore below the ICP-OES detection limit (Straka et al., unpublished results).

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Mesurer la consommation en sucre des larves dans des micro-colonies de bourdon: une nouvelle méthode prometteuse pour suivre les flux de nourriture chez l'abeille

Technique de marquage / lanthanide / glucides / insecte / ICP-OES

Messung des Zuckerverbrauchs von Larven in Hummel-Minivölkern: Eine vielversprechende neue Methode um die Ernährungsökonomie bei Bienen zu verfolgen

Markierungstechnik / Lanthanide / Kohlenhydrate / Insekten / ICP-OES

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