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Galenics: studies of the toxicity and distribution of sugar substitutes on *Apis mellifera*

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Abstract – The aim of this study was to find a substitute to sugar water in medicinal treatments of honey bee colonies with the same properties but without being ingested by bees or being toxic to them. Tylose MH, sorbitol and glycerol were tested for their attractiveness to *Apis mellifera*, their application ability, toxicity via individual application and distribution in small groups respectively a small colony. Neither of the substances proved attractive or toxic. All had good application ability except sorbitol. Glycerol showed the best distribution compared to tylose and sugar water (chi-square test, $f=1$, $P\leq 0.01$). So the distribution of glycerol within a colony was shown by macro-computed tomography: it was quick and even (comparing density values before and after treatment, Mann–Whitney rank sum test, $P\leq 0.001$). Glycerol can be recommended as a substitute for sugar water in medicinal treatments. Yet, its use with active ingredients should be investigated for combinatory effects.

galenics / sugar substitutes / *Apis mellifera* / distribution / toxicity

1. INTRODUCTION

Honey bee populations have recently faced threats to their survival. Worldwide, the number of colonies was reduced by *Varroa destructor* infestations since the second half of the last century (Rademacher 1990). US beekeepers were further affected by Colony Collapse Disorder (CCD) since 2006 (van Engelsdorp et al. 2009; Ellis et al. 2010). Varroosis seems to be one of the causes of CCD. Over many years, “hard” chemicals such as pyrethroids (Bayvarol®/ Apistan®) and coumaphos (Perizin®/ CheckMite®) were the most used synthetic acaricides against *V. destructor*. Because of the

on-going resistance of *V. destructor* against the common treatments (Milani 1999; Martin 2004; Goodwin et al. 2005; Pettis and Jadczyk 2005), there has been an urgent need to develop and to put into practice further control measures. Today, a variety of chemical, biotechnical and biological methods are used to attempt the control of mites (Rosenkranz et al. 2009). This includes “soft” chemicals such as thymol (e.g. Thymovar®/ApiLife-VAR®/Apiguard®), oxalic acid (e.g. Oxuvar®/Oxalsäure 3.5 % ad us vet®) and formic acid (e.g. Ameisensäure 60 % ad us vet®). With these methods, an effective control of Varroosis has been realised within an integrated *Varroa* control program.

There are two principal points to be considered concerning medical treatment of honey bee colonies: the toxicity of the ingredient to bees and mites and its distribution in the colony,

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which again is directly affecting the toxicity and efficacy of a substance.

A popular method of applying an ingredient into the hive is to sprinkle or trickle medicinal syrup onto the bees between the combs. This is done for Perizin[®], a systemic acaricide that the bees ingest with the syrup in the colony, so the acaricide passes into the haemolymph of the bees and kills the mites feeding on them (van Buren et al. 1992).

The trickling method is also used when applying oxalic acid (OA) into broodless colonies (Rademacher and Harz 2006). In contrast to Perizin[®], where the ingestion is essential for an acaricidal effect, oxalic acid is assumed to work as contact acaricide (Milani 2001; Aliano et al. 2006; Rademacher and Harz 2009) so ingestion is not necessary for an acaricidal effect. However, the combination of OA with sugar water is recommended because non-sugar solutions resulted in noticeably decreased effect (Charrière 2001; Charrière and Imdorf 2002; Nanetti et al. 2003). This is probably due to the high viscosity of sugar water, which allows a better adhesion on the bees and a better distribution in the colony. However, by the use of sugar water as carrier substance it cannot be excluded that the bees ingest the syrup and the acaricide. This can lead to bee mortality because of the oral toxicity of oxalic acid (Rademacher and Harz 2009).

Studies show that the distribution of oxalic acid is primarily supported by the bee-to-bee contact (Aliano and Ellis 2008). Therefore, the properties of sugar water are in fact important for better distribution. If an ingredient like oxalic acid could be combined with an agent having the same characteristics as sugar water, but neither being ingested by the bees nor being toxic to them, it would improve the treatment by making it much more tolerable for bees. Such an alternative for sugar water could be used in several present and future developed treatments of colonies, where the ingredient works as a contact poison and is applied by trickling into the hive. It also could give the possibility to use substances which have an acute oral toxicity for bees.

The aim of this study was to find a substitute to sugar water, with equal properties (high viscosity, long humidity and good distribution)

but neither being ingested by the bees nor being toxic to them.

2. MATERIALS AND METHODS

2.1. Animals

2.1.1. Laboratory trials

Worker honey bees (about 5 to 10 days old) were taken from a colony by brushing them from the combs of the brood chamber into wooden cages with food (Apifonda, Südzucker AG, Mannheim, Germany) *ad libitum*. The cages were kept in a dark room at 22 °C and 65 % R.H. during 72 h of the experiments and for 24 h prior experiment to calm down and get used to the new situation.

2.1.2. Colony trial

A small colony (*Apis mellifera carnica*) with about 3,000 individuals in winter cluster without brood was used for distribution tests with a macroCT scanner.

2.2. Chemical agents

The following three sugar substitutes were chosen as the most promising for the experiments:

- Sorbitol (D-glucitol) is a colourless and odourless liquid with a sweet taste for humans and good solubility in water (Römpp 1977). This sugar alcohol has four hydroxyl groups and is hygroscopic. It is mainly used as humectant (moisture conditioner) on printing rolls, in leather and tobacco and in writing ink to ensure smooth flow (Merck 1989; Römpp 1977). Sorbitol is known to cause no bee mortality and tasteless for bees (von Frisch 1934).
- Glycerol (1,2,3-propantriol) is a colourless, odourless, viscous liquid with a sweet taste for humans (Römpp 1977). It has three hydrophilic hydroxyl groups that are responsible for its solubility in water and its hygroscopic nature. Glycerol is widely used in pharmaceutical formulations. In the food and cosmetic industry, it is mainly used as a humectant in tobacco, paints, shoe polish, etc. (Römpp 1977). Glycerol is known to cause no honeybee mortality (von

Frisch 1934). Furthermore, it is a natural component of honey (Laub and Marx 1987; Huidobro et al. 1993) and is used as a food additive E422 with no defined acceptable daily intake (ADI) value (Die Verbraucher Initiative e.V 2000).

- Tylose MH (hydroxyethyl methyl cellulose) is a colourless, odourless and tasteless viscous liquid. The term tylose comprises an assortment of cellulose ethers with low to high viscosity derived from cellulose (Römpf 1977). Common to these cellulose ethers is the methoxylation. Additionally, the conversion can be achieved with ethylene. The result is the final product methyl hydroxyethyl cellulose (MHEC) known as tylose MH (SE Tylose GmbH & Co. KG, 2012). Tylose has good solubility in water but can form clear films (Merck 1989). It is used as thickening agent in cosmetics, as binder and stabilizer in foods, as a suspending agent in pharmaceutical industry and as an adjustment of viscosity (Merck 1989; Römpf 1977). Tylose has not been tested on honeybees so far, but due to preliminary tests we assumed it to be nontoxic.

Following concentrations were used:

- Tylose MH 0.5 %, 1 %, 3 %
- Sorbitol 7 %
- Glycerol 85 %

The substances were provided by the Department of Pharmacy (Freie Universität Berlin).

2.3. Treatment

2.3.1. Laboratory trials: properties and toxicity of the sugar substitutes in single bee treatment

In this experiment, the attractiveness for bees, the application ability and the toxicity of the substances were analysed.

To check its attraction, the bees received a drop of 5 μL of the solution on the antennae after 5 h of starvation. If the bee showed no response (extension of the proboscis, PER), it was applied directly under the proboscis. Only if the solution was not ingested was it considered as unattractive (de Brito Sanchez 2011).

For the dermal application, 5 μL of the solution was applied onto the abdomen of the bee (Figure 1). The control group received sugar water 50 % (w/w) respectively (resp.) distilled water. For each substance and concentration, three cages of ten bees with one replicate were prepared. The dosage per bee and the number of bees treated are listed in Table I.

During treatment, properties like viscosity, humidity and distribution of the substance on the cuticula and its adhesiveness were investigated. After the treatment, the bees were kept in groups of ten in new cages with food *ad libitum*. The grooming behaviour of the bees was observed for half an hour. To determine the toxicity of the substances, dead bees were counted and removed 24, 48 and 72 h after application.

2.3.2. Laboratory trials: distribution of the sugar substitutes in small bee groups

Due to the results of the single bee treatment experiment, only tylose MH 1 % and glycerol 85 % proved to be suitable for the distribution experiments. To achieve a more realistic simulation of substance distribution in the colony, the cages were equipped with a piece of comb that reached from wall to wall only leaving space beneath the comb. This allowed the bees to switch to the other comb side and distribute the substance by their activity. For every substance, 100 bees were introduced into a prepared cage.

To make the dispersion visible, a yellow dye (Procion® MX Dye 004 Lemon Yellow, Rupert, Gibbon & Spider, Inc., Healdsburg, California) was added in the ratio of 0.2 g/mL substance. On every side of the comb, 5 drops of 100 μL solution were trickled through holes in the top of the cage onto the bee clusters. Altogether, a total amount of 1 mL solution was applied to 100 bees. After treatment, the animals were provided with bee fondant (Apifonda, Südzucker AG, Mannheim, Germany) and water *ad libitum*.

After 24 h, the distribution of colour residues on every single bee was determined by examining its body surface under the binocular. The degree of colour coverage on the bee's surface was classified by establishing a ranking from 0 to 5 (0=no colour, 5=total covering). Colour residues on the combs and the amount of the solution that dropped down in the cages where the dishes were placed in were estimated as well.



Figure 1 Topical application of solution on the abdomen

2.3.3. Colony trial: distribution of the sugar substitute glycerol in a small colony unit

Internal structures of a bee hive can be demonstrated by computed tomography (Greco 2010). For visualising in hive distribution of the substance combined with the contrast agent Unilux (Iopamidol, 370 mg Iod/ml), a macroCT scanner (Xvision, Toshiba) was used (Figure 2). With a helical scanner, a distance of 250 mm was examined to make sure the colony was captured completely. The CT images were reconstructed with a slice thickness of 2 mm (technical data see Table II). For visualisation via 2D images and data analysis, we used the software eFilm™ Lite™ (Merge™ Healthcare 2008). The 2D images allow

measuring the density of individual bees in Hounsfield Units (HU). This density is directly related to the amount of solution applied to the surface of the bee's bodies. In a defined area of 100 cm² in the central area of the comb as well as in the boundary area of the bee cluster, the density of single bees was measured over three combs ($n=144$). The measurements were conducted before application (control) and 10 min resp. 30 min after applying glycerol. Only bees placed parallel to the macroCT sectional plane were quantified. The density values of the bees were transformed into a ranking from 1 to 5 (1=low density of -400 to -300 HU, 5=high density of 0 to $+100$ HU).

The total dosage of radiation for the scan of the entire bee colony was 249.8 mGy, spread over the helix distance of 250 mm (125 slices of 2 mm

Table I. Substances tested for their toxicity and properties on individual bees

Substance	Concentration [%]	Amount/bee [μ l]	Dosage/bee [μ g]	Number of bees
Tylose MH	0.5	5	2.5	60
Tylose MH	1	5	5	60
Tylose MH	3	5	15	60
Sorbitol	7	5	3.5	40 ^a
Glycerol	85	5	425	60
Sugar water	50	5	250	60

^a Due to its properties, sorbitol has been rejected after testing on 40 bees



Figure 2 Bee colony in the macroCT scanner

thickness). Compared to the dosage of 500 mGy reported by Kanao et al. (2003) for biological effects in *Drosophila melanogaster*, this dosage can be considered to be without harm to bees.

The contrast agent Unilux (dosage 0.5 $\mu\text{L}/\text{bee}$) as well as a mixture of Unilux (0.5 $\mu\text{L}/\text{bee}$) and glycerol 85 % (4.5 $\mu\text{L}/\text{bee}$) were tested for bee toxicity in the laboratory prior to trickling the colony as described in Sections 2.1 and 2.3.

Table II. High resolution protocol for *A. mellifera* colony scanning

Parameter	High resolution protocol
Slice thickness	2 mm
Pitch	2.5
Helix	250 mm
Peak X ray voltage	120 kV
X ray tube current	80 mA
Total scan time	75 s
Matrix	512x512
Scan field of view	large
Display field of view	480 mm
Window width	1000
Window level	-300
Total scan dosage	249.8 mGy

2.4. Data analysis

The number of dead bees in the test and control group as well as the degrees of colour coverage on the bees was compared with the chi-square test (Microsoft® Excel 2002). The density values of the bees in the small colony unit were analysed with Mann–Whitney rank sum test (MWU test, Sigma-Stat®3.0).

For all tests, a difference was considered to be significant when the *P* value obtained was lower than 0.05.

3. RESULTS

3.1. Laboratory trials: properties and toxicity of the sugar substitutes in single bee treatment

3.1.1. Attractiveness

None of the substances was attractive to bees. Even when applied directly under the proboscis, the bees refused to ingest any of the substances.

3.1.2. Properties in individual application

The viscosity of all substances was high enough to stay on the bees after application except for sorbitol. The dispersion of glycerol and tylose MH on the cuticle of the bees was good, even better than sugar water. Tylose MH and especially glycerol formed a wet film around the abdomen. The characteristics of the substances are summarised in Table III. Glycerol showed the best properties after application on the individual bee: it dispensed well and stayed moist for longest. Bees appeared darker and their coat looked soggy. All concentrations of tylose MH showed a better dispersion than sugar water, but dried very fast on the bee's surface. The dried tylose MH film peeled off, when the bees groomed themselves. This happened especially with the 3 % solution. Sorbitol did not dispense well on the bee's surface: it stayed in form of a drop, which often rolled off the bees.

Table III. Properties of the substances after dermal application

Substance	Humidity	Dispersion	Adhesiveness
Glycerol 85 %	high	very good	very good
Sorbitol	not assessable	stays in form of drops	drops roll off
Tylose MH 0.5 %	low (fast drying)	good	good, but peels off after drying
Tylose MH 1 %	low (fast drying)	very good	good, but peels off after drying
Tylose MH 3 %	low (fast drying)	very good	good, but peels off after drying
Sugar water	medium	good	good

3.1.3. Effect on grooming behaviour

For all substances, increased self grooming after the application was observed (Table IV). The bees tried to clean off the substances with their hind legs. Ingestion was not observed except for sugar water. Social grooming only occurred after application of sugar water or sorbitol.

3.1.4. Toxicity

After individual dermal application, bee mortality was not increased and acute toxic effects were not observed in any of the tested groups compared to the controls (sugar water/distilled water, chi-square test, $f=1$, $P \geq 0.05$, Table V).

3.2. Laboratory trials: distribution of the sugar substitutes in small bee groups

3.2.1. Distribution on the bee in small groups

Glycerol 85 % and tylose MH 1 % showed the best properties of all tested substances in the individual application and were chosen for the distribution experiments in small units.

Bees treated with coloured glycerol had the highest amount of animals with high ranking numbers between 3 and 5 (mean 3.315; Figure 3; 0=no colour, 5=total covering). In contrast most of the bees treated with tylose MH reached ranking numbers between 0 and 3 (mean 1.298).

Bees treated with sugar water solution had the lowest amount of individuals covered with colour. Most bees received rankings between 0 and 2 (mean 0.967). Significant differences could be found between glycerol and sugar water treatment (chi-square test, $f=1$, $P \leq 0.001$), tylose MH and sugar water treatment ($P \leq 0.01$) and glycerol and tylose MH treatment ($P \leq 0.001$).

After drying, glycerol formed smooth and powdery residues which had a good adhesiveness on the bees. The crystalline residues of tylose MH were much rougher and peeled off. They were mainly found on the metatarsi of the hind legs and on the veins of the wings. Sugar water formed relatively big crystals which were easily groomed off.

3.2.2. Residues on equipment

The amount of residues on combs and bees and the loss of substance in the dishes were estimated in relation to the total amount of 1 mL

Table IV. Grooming behaviour observations for half an hour after dermal application

Substance	Selfgrooming	Social grooming
Glycerol 85 %	yes (strong)	no
Sorbitol 7 %	few	few
Tylose MH 0.5 %	few	no
Tylose MH 1 %	yes	no
Tylose MH 3 %	yes (strong)	no
Sugar water	yes	yes

Table V. Rate of mortality 72 h after dermal application

Substance	Mortality 72 h after dermal application	
	n	%
Tylose MH 0.5 %	3	5
Tylose MH 1 %	2	3.33
Tylose MH 3 %	1	1.67
Sorbitol 7 %	0	0
Glycerol 85 %	1	1.67
Sugar water	3	5
Distilled water	0	0

solution applied (Table VI): the highest loss and also the highest amount of residues on the equipment could be found for tylose MH, where only 1/4 of the solution was found on the bees and about 3/4 was left on the comb and in the dish. Glycerol had few residues on equipment and little loss. Due to its good adhesiveness about 2/3 of the solution stayed on the bees. Sugar water had the lowest adhesiveness on bees; the coverage of the comb was lower than for tylose MH (Figure 4). Little loss could be found in the dish.

3.3. Colony trial: distribution of the sugar substitute glycerol in a small colony unit

In the laboratory, the contrast agent Unilux as well as the mixture of Unilux and Glycerol showed no toxic effects. Seventy-two hours after application, the bee mortality in both test groups was not significantly different from the control group (χ^2 test, $P \geq 0.05$).

The distribution of glycerol in the colony after topical application was demonstrated by macroCT scanner (Figure 5). The measurements of the control bees achieved a mean density value of -219.77 ± 93.3 HU and accordingly in the ranking a mean value of 2.3. After application of glycerol (10 min), the density value increased to a mean value of -116.66 ± 73.91 HU resp. 3.3 in ranking, significantly different from the controls (MWU test, $P \leq 0.001$; Figures 6 and 7). The mean value of the bees in the central area of the combs (-114.1 ± 81.19 HU) was comparable to the bees in the boundary area with a mean value of -119.22 ± 65.45 HU, see Figure 6. Thirty minutes after application, the mean value was -118.76 ± 67.8 HU and 3.3 in the ranking, significantly different compared to the controls (MWU test, $P \leq 0.001$) but not

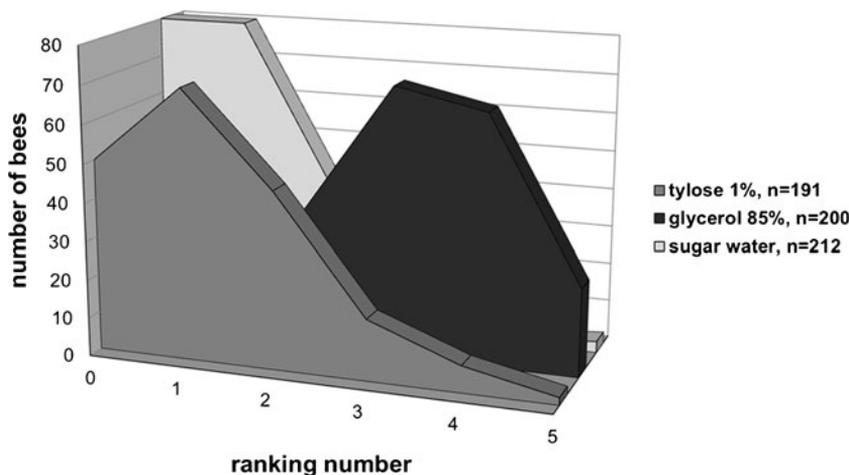


Figure 3 Frequency distribution of the ranking classification (0=no colour, 5=total covering) of bees treated with tylose MH 1 %, glycerol 85 % or sugar water

Table VI. Residues of the substances

Substance	Residues on equipment	Loss in dishes	Residues on bees
Glycerol 85 %	17 %	17 %	66 %
Tylose MH 1 %	45 %	30 %	25 %
Sugar water	30 %	15 %	19 %

significant different from the values achieved after 10 min (Figures 6 and 7).

4. DISCUSSION

The applicability of the substances as carriers for medicinal ingredients is discussed under the following aspects: attractiveness to bees, properties in individual application, effect on grooming behaviour, toxicity to honey bees, residues on equipment and distribution on the bee and in the colony.

Since none of the substances was attractive to bees an undesired ingestion of a medicinal ingredient combined with one of them can be excluded assuming that the ingredient itself is

unattractive. Even combined with sugar water (25 %), glycerol would not be ingested by honey bees (von Frisch 1934).

In the individual application experiment, glycerol had the best properties and would be most suitable for colony treatment: it stayed moist for the longest, the bees were wet for more than half an hour. There is a long period of time when the transfer of the substance from bee to bee is possible and many individuals get in contact with the ingredient. The second substance tested—tylose—peeled off after drying, especially the 3 % solution so that an application in colony treatment for this concentration cannot be recommended. This is also the case for sorbitol which proved unsuitable for application on the bee due to bad adhesiveness and dispersion. If used in medical treatment, a great amount of it will get lost after a short time.

Self grooming was increased for all applied substances. This is a desired behaviour which provides a good distribution of the solution on the bee's surface. Social grooming was only observed for sorbitol and sugar water. When bees were grooming one another with their proboscis, the substances were probably ingested. An accidental ingestion of any of the

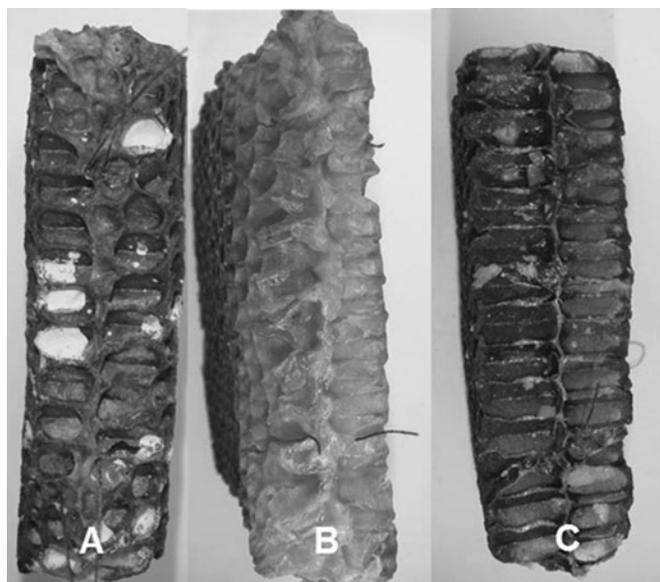


Figure 4 Combs covered with colour-residues of tylose MH (a), glycerol (b) and sugar water solution (c)

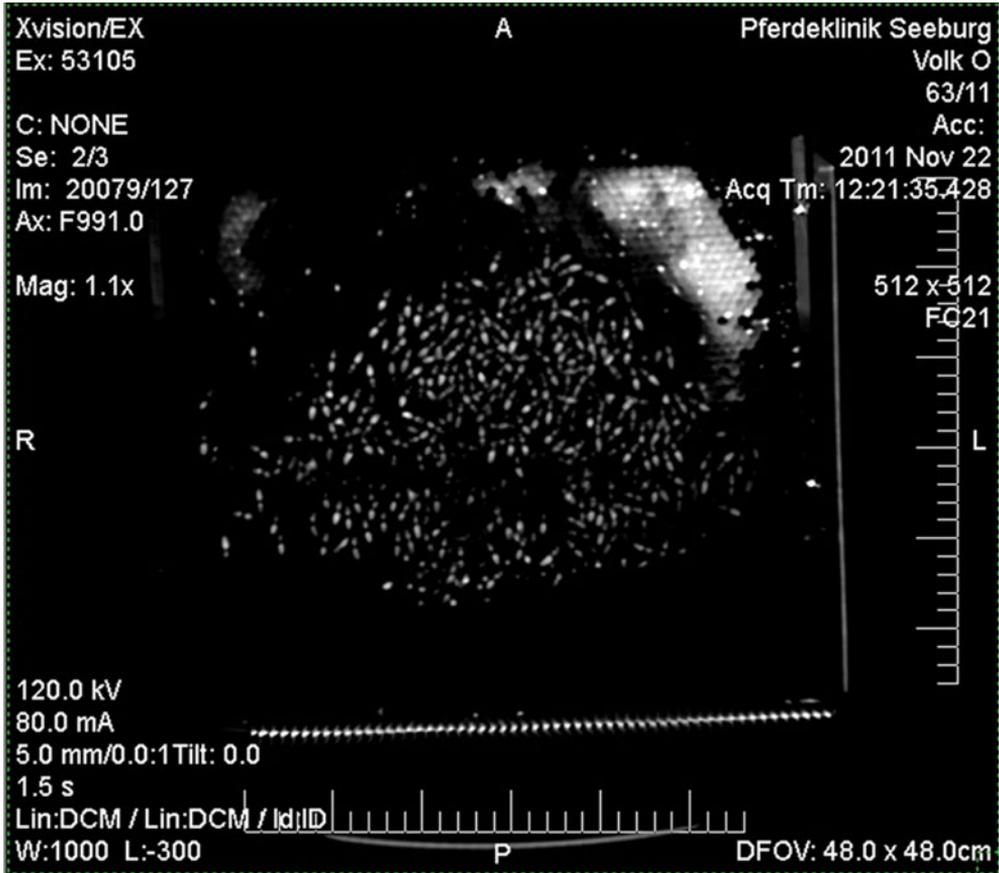


Figure 5 MacroCT scanner image: bees on the comb after application of glycerol combined with Unilux

substances in the groups without social grooming can be nearly excluded.

Glycerol 85 % and tylose MH 1 % were chosen for the distribution experiments in small

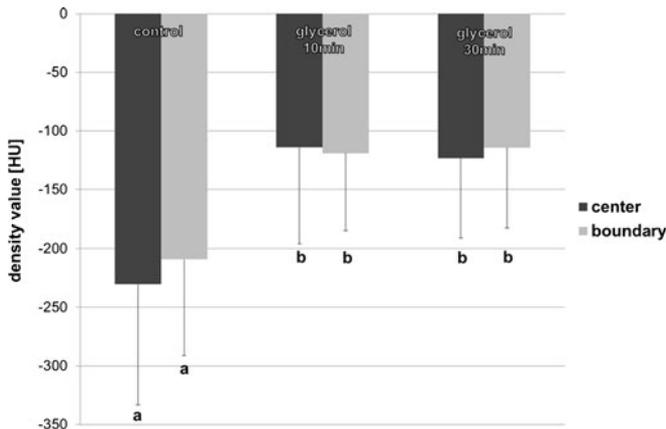


Figure 6 Mean density of bees in the colony (significant differences marked by different letters)

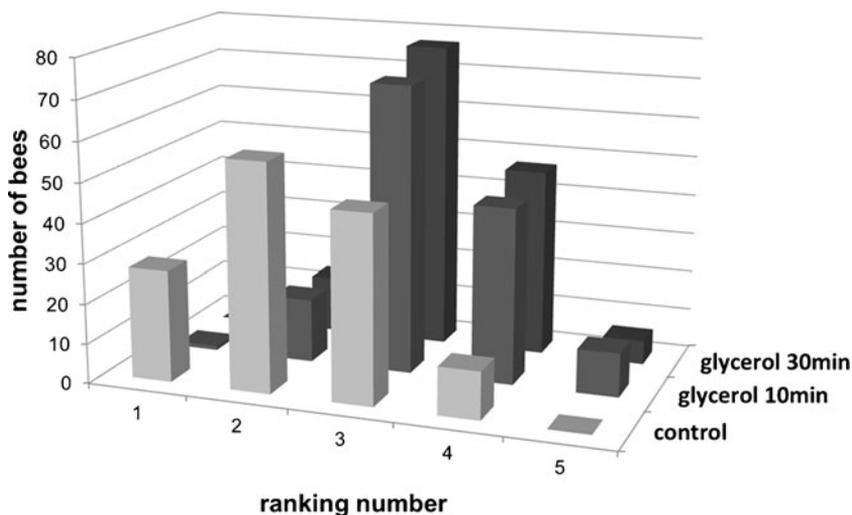


Figure 7 Frequency distribution of the ranking classification of bees in the colony

units, because in individual application they showed the best properties of all tested substances and achieved the defined requirements as a substitute for sugar water: they had no toxic effect and realised a better distribution on the surface of honey bees than sugar water.

The results of the distribution in small bee groups resemble the observations of the application properties on the individual bee. Glycerol had the best distribution. Due to its high viscosity and humidity most of the animals got in contact with the solution even long after the application so that on almost every bee traces of the substance could be found. The residues of tylose MH peeled off and a high amount was found on the equipment. This supports the observations in the individual application experiment. Due to its bad adhesiveness properties, it can be assumed that the bees were able to groom off a great amount of the tylose MH solution. Sugar water showed the worst distribution. It formed relatively big crystals which were easily groomed off by the bees. However, little loss could be found in the dish. The attractiveness of sugar water could have motivated the bees to increased grooming behaviour and ingestion of the substance. It is assumed that part of the substance was lost due to oral uptake. This is an important

aspect considering medical treatments against Varroosis and shows the need for an alternative: for example, oxalic acid, which has a toxic effect on honeybees when ingested (Rademacher and Harz 2009), is combined with sugar water to achieve a better acaricidal effect (Charrière 2001; Charrière and Imdorf 2002; Nanetti et al. 2003).

For all aspects, glycerol proved the most suitable substance and was chosen for colony distribution via macroCT. Combined with the contrast agent, glycerol has a high density in the X-ray. The results of the roentgenoscopy showed high density values for individual bees in the test colony, much higher than the control measurements. This means the substances can be detected on the bees. A good distribution was already achieved after 10 min. This could be documented in the central as well as in the boundary area. Thereby the macroCT investigation demonstrated a quick and uniform distribution of glycerol.

5. CONCLUSION

In general, it can be concluded that glycerol 85 % is most suitable as a sugar water substitute. It had the best distribution properties and stayed moist long enough to assure a

prolonged contact of the bees with the substance. After drying the powdery consistency of the residues might contribute to a better distribution and adhesiveness on the bees. An oral uptake can be excluded. Possible residues of glycerol after colony treatment can be considered as harmless.

Glycerol distributes quickly and uniformly in the colony and can provide a good dispersion of medicinal active ingredients as a carrier substance. However, glycerol actually was not tested in combination with a medicinal ingredient. In any case, it must be clarified if glycerol can be combined with an active ingredient without showing combinatory toxic effects to the bees and to make sure that its effectiveness is not impaired by the combination with glycerol.

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Galénique: études de la toxicité et de la distribution de substituts du sucre sur *Apis mellifera*

galénique / substance de substitution / abeille / effet toxique / mode de distribution / traitement sanitaire

Galenik: Untersuchungen zur Toxizität und Verteilung von Zuckerersatzstoffen auf *Apis mellifera*

Galenik / Zuckerersatzstoffe / *Apis mellifera* / Verteilung / Toxizität

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