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**Effect of Hydroxymethylfurfural (HMF) on
mortality of artificially reared honey bee larvae
(*Apis mellifera carnica*)**

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Abstract

Hydroxymethylfurfural (HMF) is a heat-formed, acid-catalyzed contaminant of sugar solutions, such as inverted sugar syrup, high fructose corn syrup (HFCS) or honey, who all find their way into honey bee feeding. As HMF was noted to be toxic to adult honey bees, we investigated toxicity of HMF towards larvae. Therefore we exposed artificially reared larvae to a chronic intoxication over 6 days, with 6 different concentrations (5, 50, 750, 5000, 7500 and 10000 ppm) of HMF. Mortality was assessed from day 2 to day 7 (d7) and day 22 (d22). Concentrations ranging from 5 to 750 ppm HMF did not show an influence on larval or pupal mortality, compared to controls ($p > 0.05$; Kaplan-Meier analysis). Concentrations of 7500 ppm or higher caused a larval mortality of 100%. An experimental LC_{50} of 4280 ppm (d7) and 2424 ppm (d22) was determined. The calculated LD_{50} was 778 μg HMF on d7 and 441 μg HMF on d22. Additionally we exposed adult honey bees to high concentrations of HMF and compared them to our larval results. On d7 larvae are much more sensitive against HMF than adult honey bees after 6 days of feeding. However, measured on d22, adults show a lower LC_{50} , which indicates that they are more sensitive than larvae. Note that d22 larvae ingest HMF with their food only for 7 days, as pupae do not consume. Hence apparently their tolerance towards HMF increases compared to adult workers. As toxicity of HMF against honey bees is a function of time and concentration, our results indicate that HMF in supplemental food will probably not cause great brood losses. Yet sublethal effects might reduce resistance against pesticides, parasites and diseases and decrease fitness of the whole colony.

Keywords: honey bee, HMF, in vitro rearing, chronic intoxication, supplemental food

Zusammenfassung

Hydroxymethylfurfural (HMF) ist eine chemische Verbindung, die unter dem Einfluss von Hitze und saurem pH-Wert in zuckerhaltigen Substanzen, wie Invertzuckerlösung oder Honig entsteht. Da diese Lösungen bei der Bienenfütterung eine Rolle spielen können und HMF einen toxischen Effekt auf adulte Bienen hat, wollten wir die Wirkung auf Bienenlarven untersuchen. Dazu setzten wir künstlich aufgezogene Larven einer chronischen Vergiftung mit 6 verschiedenen Konzentrationen HMF über 6 Tage aus. Die Mortalität wurde von Tag 2 bis Tag 7 (d7) und am Tag 22 (d22) protokolliert. Konzentrationen von 5 bis 750 ppm HMF zeigten dabei, verglichen mit der unbehandelten Kontrolle, keine Unterschiede in ihrer Mortalität ($p > 0.05$; Kaplan-Meier Analyse). Konzentrationen von 7500 ppm oder höher resultierten hingegen in einer Larvensterblichkeit von 100%. Die LC_{50} lag bei 4280 ppm HMF an d7 und 2424 ppm HMF an d22, die LD_{50} lag bei 778 μg HMF an d7 und 441 μg HMF an d22. Zusätzlich haben wir adulte Bienen einer hohen Konzentration HMF ausgesetzt und die Ergebnisse mit denen der Larvenversuche verglichen. An d7 waren die Larven viel empfindlicher gegenüber dem Einfluss von HMF, verglichen mit den Adulten. An d22 hingegen, waren die Adulten empfindlicher gegenüber den Larven. Man beachte, dass Larven HMF mit dem Futter nur über 6 Tage aufnehmen, da sie im Puppenstadium keine Nahrung konsumieren. Dies scheint der Grund zu sein, warum die HMF-Toleranz der Larven gegenüber den Arbeiterinnen an d22 zunimmt. Die Toxizität von HMF auf Honigbienen scheint eine Funktion aus Zeit und Konzentration zu sein, daher deuten unsere Ergebnisse darauf hin, dass HMF in zugefütterten Zuckerlösungen wahrscheinlich zu keinen großen Brutverlusten führt. Allerdings können sublethale Effekte zu einem geringeren Widerstandsvermögen gegen Pestizide, Parasiten und Krankheiten führen und in weiterer Folge die Gesundheit des gesamten Stockes beeinflussen.

1. Introduction

Honey bee nutrition plays a vital part in developing and maintaining healthy and strong colonies. Next to protein, which is necessary for brood production, carbohydrates are essential, as they provide energy for all activities within the hive and during foraging. Honey bees naturally obtain carbohydrates by collecting honeydew or nectar. In times when natural sources are limited or harvesting honey has removed a colonies supply of carbohydrates, supplemental feeding by beekeepers is necessary [Haydak, 1970; Brodschneider and Crailsheim, 2010] and quite common. Usually sucrose solution, inverted sugar syrup or other syrups, such as starch syrup or high fructose corn syrup (HFCS) are used for that purpose [Jachimowicz and El Sherbiny, 1975; Le Blanc et al., 2009; Brodschneider et al., 2010; Brodschneider and Crailsheim, 2010].

Since it was found that some sugars, for example galactose, mannose and lactose are toxic to honey bees in certain concentrations [Barker and Lehner, 1976; Barker, 1977], these do not find their way into supplemental feeding.

Yet another substance that is toxic to bees can be found in syrups applied for colony nutrition: Hydroxymethylfurfural (HMF), a chemical compound, forming from carbohydrates, especially fructose, under thermal and/or acid-catalyzed decomposition. It is found in many sugar containing food products, like invert sugars, juices, jams, syrups and honey [Jachimowicz and El Sherbiny, 1975; ALabdeen Makawi et al., 2009, LeBlanc et al., 2009]. As mentioned above, some of those products are used supplemental honey bee feeding, such as inverted sugar syrup or HFCS. Sometimes even non used honey is brought back to the hive as supplemental food. As a consequence, HMF content of those products is of great interest to beekeepers. Three methods are commonly used to detect HMF in sugar solutions, which have been tested to deliver quite similar results [Bogdanov, 2002], the HPLC method [Jeuring and Koppers, 1980; Kim and Richardson, 1992; Risner et al., 2006] and the two spectrophotometric methods of Winkler [1955] and White [1979].

Contamination of honey and different sugar solutions with HMF has been investigated very well. Le Blanc et al. [2009] investigated HMF content of commercially available HFCS of different brands and found concentrations ranging between 3.1 and 28.7 ppm HMF for fresh syrups. They also analyzed formation of HMF in several samples of HFCS under different temperature conditions. Results showed that constant high temperatures can cause high levels of HMF, especially in acid-catalyzed HFCS. For example, a temperature of 49°C can cause a formation of more than 200 ppm HMF over a period of 36 days. Some samples exposed to

69°C for the same time, obtained HMF values higher than 30.000 ppm HMF. Ruiz-Matute et al. [2010] found HMF concentrations ranging from 26.91 to 102.31 ppm in samples of HFCS. Analysed samples supplied by manufacturers showed lower HMF-levels than those supplied by beekeepers, suggesting the former have been fresh and properly shelved. The syrup-samples containing the highest amounts of HMF were stored in metal tanks, standing outside unprotected from sunlight and hence could be exposed to permanent high temperatures, especially during summer months. Van der Zee and Pisa [2010] investigated HMF content of (Fructo-Bee®) inverted sugar solution. Samples were leftovers of winter feeding, provided by beekeepers that had suffered of high colony losses over the winter. They found HMF values up to 466.2 ppm in samples with a “best before” date nearly 4 years after analysis.

Fresh honey usually contains no or low amounts of HMF [Bogdanov et al., 1999]. Due to this fact, HMF content has become one of the most important criteria in honey quality evaluation [White, 1994]. On the European market honey must contain less than 40 ppm HMF when placed on the market [Council Directive relating to honey, 2002]. Due to improper storage, honey, which naturally shows an average pH of 3.91 for floral and 4.45 for honeydew honey [Doner, 1977], can develop a high amount of HMF during storage, just like HFCS, which is quite similar in sugar composition and pH-value [Ruiz-Matute et al., 2010]. Tosi et al. [2001] investigated the effect of short term heating on HMF concentration of different honey samples and found that an exposure of 60 seconds at 100°C caused an increase from 3.9 to 10.1 ppm HMF. Karabournioti and Zervaki [2001] exposed honey of several botanic origins to different temperatures for 24 hours. They found HMF concentrations ranging from 1.95-29.20, 2.25-32.60, 4.35-39 ppm HMF, at temperatures of 35, 45 and 55°C, respectively. Exposure to 75°C for 24 hours resulted in HMF concentrations from 43.40 to 226.35 ppm HMF. ALabdeen Makawi et al. [2009] found high HMF concentrations in Sudan honey samples (up to 922 ppm), stored for several years.

Bailey [1966] already discovered, that honey stored for many years and therefore containing high amounts of HMF, caused increasing mortality to bees, compared to fresh honey. He also observed that HMF caused gut ulceration resulting in dysentery. The toxicity of HMF on adult workers was, among others, investigated by Jachimowicz and El Sherbiny [1975]. They showed that a level of 150 ppm HMF, solved in a mimicked inverted sugar solution consisting of glucose, fructose and sucrose, caused a significantly increased mortality within 20 days in caged bees. A concentration of 30 ppm HMF however, did cause no significant difference in mortality of adults, approving the European Union Council Directive relating to honey [2002]. Le Blanc et al. [2009], feeding HMF contaminated HFCS, reported results very close

to those of Jachimowitz and El Sherbiny and found that over a period of 26 days, 250 ppm HMF caused mortality significantly higher than any of the other (lower) treatment groups.

These studies indicate that the toxicity of HMF on adult workers is well investigated. Yet little is known about toxicity of HMF to larvae. Larval food, which is next to protein and lipids rich in carbohydrates, is produced by nurse bees after ingesting carbohydrates and pollen [Haydak, 1970; Brouwers et al., 1987; Kunert and Crailsheim, 1987]. It is likely, that HMF finds its way into larval nutrition when nurse bees consume contaminated honey or sugar syrup such as HFCS, but larval susceptibility has not been studied yet.

To test effects of chemicals, such as insecticides, on honey bee brood, different methods have been published over the years. Oomen [1992] described an in-hive method, which is an official recommended method for testing insect growth regulators, according to the guidelines of the European Union [OEPP/EPPO, 2001 and 2003]. Yet, this method may not be easily reproducible, due to changing environmental conditions and the influence of adult workers. Besides, the in-hive test supplies no individual quantitative data, as no one can determine, how much of the tested substance is ingested by larvae [Aupinel et al., 2007]. Methods developed for risk assessment towards honey bee larvae in the laboratory [Rembold and Lackner 1981; Vandenberg and Shimanuki, 1987; Peng et al., 1992; Aupinel et al., 2005; Medrzycki et al., 2010; Hendriksma et al., 2011] allow the precise determination of lethal concentration and/or dose. The quality of artificially reared bees has been investigated by Brodschneider et al. [2009] by comparing flight performance of *in vitro* reared larvae and their natural sisters. Results indicate that artificially reared bees at the natural age of flight show a similar performance as those reared in the hive.

We investigated the effect of chronic intoxication with HMF on mortality of artificially reared honey bee brood. Further we wanted to know, whether larvae are more sensitive to HMF than adults. If that is the case, supplemental feeding with HMF contaminated food could influence larval mortality and cause brood-losses as well as sublethal damage.

2. Materials and Methods

2.1 Larval material

All larvae used in the following tests, originated from three hives of *Apis mellifera carnica*, located at the Department of Zoology, Karl-Franzens-Universität, Graz.

To obtain 1st instar larvae, the queen was caged on an empty comb in a queen excluder cage in the colony for 36 hours, then was released and set back to the hive. Worker bees were able to move in and out of the cage without limitation all the time. Four days after caging, the comb was taken to the laboratory, where grafting of larvae took place on day 1 with 5-10 old larvae.

2.2 Grafting of larvae and rearing plates

To rear larvae under laboratory conditions, we adopted a method close to Aupinel [2005].

48-well culture plates were prepared to provide a convenient replacement for the natural comb. Pieces of dental roll were placed at the bottom of each well. 500 µl of 15% glycerol solution per well were used to damp the dental roll. A common plastic queen starter cup was placed at the top of the dental roll. Prior to this, the cup has been sterilized by a solution of Milton Sterilising Tablets (Milton), rinsed with autoclaved, deionised water and dried. After preparation, the entire plate was put under UV-light over night for sterilisation. Before grafting, each cup was supplied with 10 µl of diet for the first day.

First instar larvae were transferred from the comb to the queen starter cup, by using a small paintbrush. To avoid microbial contamination, brushes for grafting were sterilized with ethanol (96%) and rinsed with autoclaved, deionised water.

2.3 Control diet

Three basic diets were fed, diet A, B and C [Aupinel et al., 2005]. On day 1 and 2, diet A was given, on day 3 diet B and from day 4 to day 6 larvae received diet C. The amount (µl) per larva/day was 10, 10, 20, 40, 40, and 50, respectively. This resulted in a total amount of 170µl of food per larvae. Each diet consisted of 50% royal jelly and 50% aqueous sugar solution (w/w). The composition of these 3 sugar solutions (su) changed from 12% fructose, 12% glucose, 2% yeast extract (sugar solution A), to 15% fructose, 15% glucose, 3% yeast extract (sugar solution B) to 18% fructose, 18% glucose, 4% yeast extract (sugar solution C).

Solid components were weighed, solved in sterilised, deionised water and filled up to a certain volume (w/v). Basic diet without HMF was fed to control group.

Royal jelly used in these experiments was purchased at a local store for beekeeper supply in 50g portions and freeze stored at -70°C. Defrosted jelly was kept refrigerated at 4°C and used up within a week.

2.4 HMF- test concentration diet

Food for test groups was supplied with HMF purchased from SAFC in the following way: a highly concentrated HMF stock solution (st) of 40.000 ppm was prepared and a double concentrated (A, B or C)-sugar solution was used for preparation and diluted afterwards according to Tab. I.

For one plate (=48 larvae) 1000 mg of diet A, 2000 mg of diet B and 3000 mg of diet C, depending on testing day, were prepared freshly. Excess of food was dumped if not used. To calculate the amounts of all ingredients for test diet preparation, Tab. 1 was used.

To find a suitable concentration range, preliminary tests were carried out. The final test concentrations (tc) were set at 5, 50, 750, 5000, 7500 and 10.000 ppm HMF to allow estimation of a LC_{50} within 7 days of larval development. As we investigated chronic intoxication, HMF was given to test groups with the food from day 1 (=day of grafting) to day 6. Whereas the quantum and composition (diet A, B, C) of applied food changed over the period of feeding, HMF concentration remained constant all the time.

2.5 Feeding of larvae

Before feeding, each larva was checked for its health status. A larva was defined as healthy, when it was plump and shiny and movements of breathing pores could be observed under the binocular. A Larva that did not show any of these characteristics was considered as dead and removed for reasons of sanitation.

Larvae were fed over a period of 6 days. Feeding took place under a laminar flow to keep risk of contamination at a minimum. Amount and composition of diet changed over the feeding period, as described by Aupinel et al. [2005]. The prewarmed food was supplied once a day, at the same time as grafting on day 1 took place.

2.6 Incubation of larvae

Larvae were transferred into an incubator with a constant temperature of 34.5°C. To maintain humidity, rearing plates were placed in hermetic Plexiglass dessicators. Saturated solution of K_2SO_4 was used to keep humidity at 96% for the first 7 days. To prevent stretching larvae from dropping out, queen cups were lowered by removing the dental rolls on day 8. On the same day, plates were transferred to a dessicator supplied with saturated solution of NaCl, which kept humidity at approximately 80%. To avoid deformation of emerging adults plates were put into an upright position on day 12 [Brodschneider et al., 2009]. A thin, perforated layer of wax prevented pupae from falling out of the plate and enabled eclosion of adults.

A special plastic lid, fitting on the edges of the 48-well plates and serving as a spacer, with holes for air ventilation, allowed bees to emerge, but kept them at the plate and avoided mixing with bees from other testing groups. An Eppendorf tube (2ml) with holes at the tip was filled with honey solution (50%) and placed inside this box, to provide food for emerging bees.

Mortality of each group was noted from day 2 (d2) to day 7 (d7) and on day 22 (d22), when bees had eclosed.

2.7 Feeding of adult bees

To validate the effect of the purchased HMF adult bees were fed with 50 % sucrose solution, containing HMF in three concentrations: 2000, 4000 and 8000 ppm. The control group received pure sucrose solution. We chose sucrose, as caged bees survive best on sucrose syrup [Barker and Lehner, 1978]. Newly emerged worker bees (younger than 24 hours), originating from the same three hives as larvae, were transferred to cages slightly modified after Evans et al. [2009]. Food was provided ad libitum in Eppendorf tubes with small holes. Mortality of each group was noted daily until d22. Three replicates of 100 bees each were run per treatment group, which resulted in a total number of 1200 bees.

2.8 Mode of analysis

We performed 51 trials with 48 larvae per replicate. To calculate the median lethal concentration (LC_{50}) the linear regression of dose-response curves for each day was calculated using MS-Excel (Microsoft). The obtained LC_{50} was used to calculate the median lethal dose (LD_{50}), assuming that the total amount of 170 μ l diet was consumed by each larva. This is legitimized as those larvae that had not ingested all of the provided food by d7 were observed

to die during pupal stage. To compare results to those of other studies dealing with toxicity of chemicals on larvae, we decided to focus on mortality on d7 and d22. Survival analysis was performed with MedCalc (MedCalc Software), using the Kaplan-Meier analysis.

3. Results

3.1 Larvae

On d7, 24 hours after last administration of food, mortality of test groups receiving concentrations 5, 50 or 750 ppm HMF was noted as 6.9% (± 1.5 se; n=288), 10.1% (± 1.77 se; n=288) and 6.8% (± 1.38 se; n=336), respectively (Fig. 1). A test concentration of 5000 ppm HMF resulted in mortality of 52.4% (± 2.72 se; n=336). The two highest of tested concentrations, 7500 (n=288) and 10.000 (n=336) ppm HMF both resulted in a mortality of 100% on d7 (Fig. 1). The non-treated control group showed a mean mortality of 9% (± 1.19 se, n=576) calculated from grafting until d7. The three lowest tested concentrations (5, 50, 750 ppm) did not show a significant effect on larval mortality, compared to control group ($p > 0.05$; Kaplan-Meier analysis). However, significant different mortality could be detected between control group and the 5.000 ppm, 7.500 ppm and 10.000 ppm HMF treatment group ($p < 0.0001$; Kaplan-Meier analysis). Differences ($p < 0.05$; Kaplan-Meier analysis) in mortality between the 5.000 and 10.000 ppm treatment group and control group were already spotted on d2, whereas the 7.500 ppm treatment group showed a significant difference firstly on d3 (Fig.1).

Larval mortalities until d22 for concentrations 5, 50 and 750ppm HMF were 26.4% (± 2.60 se), 30.2% (± 0.0271 se) and 32.1% (± 0.0255 se), respectively. Control group mortality was noted at 28.1 (± 0.0187 se) %. No significant difference was detected between mortality of control and the three lowest test groups ($p > 0.05$, Kaplan-Meier analysis). The treatment group receiving 5000ppm HMF showed a mortality of 87.5% (± 0.0180 se) on day 22 (see Fig.1), which was significant higher than that controls ($p < 0.05$; Kaplan-Meier analysis).

A linear regression of $y = 0.0101x + 6.7702$ for d7 and $y = 0.0083x + 29.878$ for d22 was calculated (see Tab. II), whereby y = mortality and x = ppm HMF. Linear regressions and coefficients of determination for d2 to d7 as well as d22 are presented in Tab. II.

The estimated LC_{50} of HMF strongly depends on the duration of experiments (Tab. II). For example, a LC_{50} of 4280 ppm (d7) and 2424 ppm (d22) HMF was determined. According to a total quantity of 170 μ l of food per larva and the calculated LC_{50} , we assessed an LD_{50} for a

chronic intoxication over 6 days. This calculated LD₅₀ was 778 µg HMF on d7 and 441 µg HMF on d22.

3.2 Survival test of adult bees

Measured on d7 adult worker control group and 2000 ppm HMF test group both showed a mortality of 0.7%, with a standard error of ± 0.470 , each (Fig. 2). The 4000 ppm HMF test group evinced a mortality of 3% (± 0.985 se) and treatment group receiving 8000 ppm HMF showed a mortality of 5% (± 1.26 se) (Fig. 2). At the end of the test on d22, mortality of untreated control group was 6.7% (± 1.44 se), whereas all bees feed with 4000 ppm HMF in sucrose solution were dead on d20 (Fig. 2). Treated bees fed with 8000 ppm HMF showed a mortality of 100% on d15. Within the test group receiving 2000 ppm HMF, a mortality of 67% (± 2.71 se) was noted on d22 (Fig. 2).

Caged adults of different treatment groups did not show differences in mortality during the first 5 days. On d6 mortality of the 4000 ppm HMF treatment group started to be significantly higher ($p=0.011$, Kaplan-Meier analysis) than that of control group. Mortality of workers fed with 8000 ppm HMF started to become significantly higher than that of controls on d7 ($p=0.0014$, Kaplan-Meier analysis). The treatment group that received 2000 ppm HMF showed a significant different mortality, compared to control, firstly on d16 ($p=0.0022$, Kaplan-Meier analysis).

Mortality data obtained throughout the whole test period were used to determine the linear regression, a LC₅₀ and the coefficient of determination (R^2), same as for larvae. Data are presented in Tab. II. For adult workers we determined a LC₅₀ of 82778 ppm HMF for d7 and a LC₅₀ of 1843 ppm HMF on d22, no LD₅₀ was calculated because individual consumption is unknown.

3.3 Comparison of larvae and adults

Data of linear regression, coefficient of determination and LC₅₀ for both, larvae and adults are shown in Tab. II. The R^2 indicates that reliability of shown values is dependent on the duration of intoxication. Compared on d7, larvae show a much higher sensitivity on intoxication with HMF than adults. Until d22 however, workers are slightly more sensitive than larvae.

Chosen dose-response curves of larvae and adults are shown in Fig. 3. Mortality of larvae dramatically increases between d2 and d4 (Fig. 3. a), whereas that of workers increases on d8

(Fig. 3. b). As larval and adult mortality differ from d3 to d7, with a mortality indicating that larvae are more sensitive to HMF before d7 (see Fig. 3 c and d), mortality of adults and larvae converge until d22 (see Fig. 3 a + b).

3.4 Tables and Figures

Table I: Preparation of the three different test-diets according to different HMF concentrations: amount of sugar solution (su) and royal jelly (RJ) is constant in different concentrations of HMF test food, whilst quantity of stock solution (st) and aqua dest. change within concentration range. Stock solution contains 40.000 ppm HMF (=stock concentration, sc). Test concentrations (tc) were 5, 50, 750, 5000, 7500 and 10.000 ppm HMF.

day	diet	stock solution (st) [μl]	royal jelly (RJ) [mg]	sugar solution (su) [mg]	aqua dest. [μl]	diet per plate [mg]
1,2	A	1000/(sc/tc)	500	250	1000-(st+RJ+su)	1000
3	B	2000/(sc/tc)	1000	500	2000-(st+RJ+su)	2000
4,5,6	C	3000/(sc/tc)	1500	750	3000-(st+RJ+su)	3000

Table II: Linear regression (y), coefficient of determination (R^2) and LC_{50} of larvae and adult bees from d2 to day 22. As day 1 was day of grafting and first application of HMF, inquiry of mortality started on day 2. To minimize the influence of handling on development of pupae, larval mortality was not assessed from d8 to d22.

	larvae			adults		
day	linear regression	R^2	LC_{50}	linear regression	R^2	LC_{50}
d2	$y = 0.0008x + 1.7761$	0.2456	60280	$y = 0.0001x + 0.0667$	0.2136	499333
d3	$y = 0.0053x + 0.9472$	0.6882	9255	$y = 0.0001x + 0.0667$	0.2136	499333
d4	$y = 0.0088x + 0.6175$	0.8719	5612	$y = 0.0001x + 0.0667$	0.2136	499333
d5	$y = 0.0098x + 1.5034$	0.8905	4949	$y = 0.0001x + 0.2$	0.28	498000
d6	$y = 0.0101x + 3.6835$	0.9171	4586	$y = 0.0002x + 0.6667$	0.1203	246667
d7	$y = 0.0101x + 6.7702$	0.9358	4280	$y = 0.0006x + 0.3333$	0.467	82778
d8	-	-	-	$y = 0.0017x - 1.2$	0.8609	30118
d9	-	-	-	$y = 0.0046x - 5.6$	0.8117	12087
d10	-	-	-	$y = 0.0081x - 11$	0.7799	7531
d11	-	-	-	$y = 0.0103x - 13.533$	0.8221	6168
d12	-	-	-	$y = 0.0119x - 14.133$	0.8667	5389
d13	-	-	-	$y = 0.0129x - 12.467$	0.917	4842
d14	-	-	-	$y = 0.0133x - 8.4$	0.9264	4391
d15	-	-	-	$y = 0.0136x - 3.4667$	0.8733	3931
d16	-	-	-	$y = 0.0136x + 1.3333$	0.8129	3578
d17	-	-	-	$y = 0.0132x + 7.4$	0.7682	3227
d18	-	-	-	$y = 0.0126x + 13.733$	0.7688	2878
d19	-	-	-	$y = 0.0119x + 20.2$	0.7448	2504
d20	-	-	-	$y = 0.0113x + 25.8$	0.6895	2142
d21	-	-	-	$y = 0.0109x + 29.2$	0.6546	1908
d22	$y = 0.0083x + 29.878$	0.7858	2424	$y = 0.0108x + 30.099$	0.6510	1843

Figure 1. Survival probability for six different concentrations of HMF and control group, noted from d1 to d7 and on d22. n=288 (groups 5, 50, 7500 ppm) each, n=336 (groups 750, 5000, 10.000 ppm) each and n=576 for control group.

Figure 2. Survival probability from d1 to d22 for caged bees treated with three different concentrations of HMF and non treated control group. n=3 replicates each, with 100 bees per replicate.

a)

b)

c)

d)

Figure 3. Dose response curves of larvae and adults, treated with different HMF concentrations. A letters indicate adults, L letters larvae. a) shows data of larvae only from d2 to d7 and d22, b) shows data of adults only from d2 to d22, c) shows data of adults and larvae from d2 to d4 and d) shows data of larvae and adults from d5 to d7.

4. Discussion

This is the first study of toxicity of HMF on honey bee larvae, using the *in vitro* standardized feeding method protocol by Aupinel et al. [2005, 2007]. Larvae treated with concentrations of 750 ppm HMF or lower, didn't show increased mortality, compared to control. In our experimental setup below 5000 ppm HMF no difference can be detected within 7 days of larval life. Larval mortality of control group was below 10% on d7, with no larvae replaced after grafting, as chronic intoxication started on d1. An estimated LC_{50} of 4280 on d7 and 2424 ppm HMF on d22 for larvae indicates that they are more resistant to HMF than we had expected, referring to HMF related data for caged workers [Jachimowicz and El Sherbiny, 1975, Le Blanc, 2009].

Little is known about the physiological effects of HMF on larvae or its metabolism. We do not know whether it causes gut ulceration as it is suggested in adult bees [Bailey, 1966] but similar mechanisms might be possible. HMF fed to larvae might be exuded with faeces or accumulated and stored in larval body. Furthermore, possible damage could be repaired during metamorphosis. Comparing our current results to published data of chronic intoxication with the pesticide dimethoate [Aupinel et al., 2007], which caused a LC_{50} lower than 10 ppm, we found that HMF is much less toxic after 6 days of oral exposure.

As royal jelly contains 7-18 % carbohydrates [Sabatini et al., 2009] one might consider that actual HMF content of our food might be higher than the stated concentrations. Yet we neglect this risk, as we purchased fresh royal jelly and storage temperature could not have induced formation of HMF. As fresh honey contains no or low amounts of HMF [Bogdanov et al.; 1999] its content in fresh royal jelly should be negligible.

Our current results for intoxication of adult workers can be compared to those of caged bees in preceding studies (Jachimowicz and El Sherbiny, 1975, Le Blanc, 2009), with the limitation that they used different sugar solutions. We used sucrose solution due to proven best longevity of adult bees [Barker and Lehner, 1978], whereas Jachimowicz and El Sherbiny [1975] used an inverted sugar solution of glucose, fructose and sucrose and Le Blanc et al. [2009] fed HFCS. Besides, both studies did not test concentrations higher than 750 ppm HMF and our test range covered concentrations up to 10.000 ppm HMF, which was legitimized by the results of our larval experiments.

Our comparison of toxicity of HMF to larvae and adults is hypothetical, not yet directly possible. Mass, consumption rate, composition of food and metabolism differ between these different development stages [Hrassnigg and Crailsheim, 2005]. Metabolism changes even in

adult bees, depending on their age and stage of age polyethism [Haydak, 1970]. For example, duration of chronic intoxication is limited to a larval feeding period of 6 days and HMF intake was limited by the rigid applied amount of food close to natural conditions [Aupinel et. al, 2005], whereas adult bees consumed food and hence HMF ad libitum. They also received HMF within their food uninterrupted until day 22, which is a much longer period and higher overall exposition than immature stages, as pupae do not consume.

In our experiments we fed HMF in various concentrations solved in typically composed food to the two developmental stages [Barker and Lehner, 1978; Aupinel et al., 2005], what allowed us to compare larvae and adults via dose-response curves. From this we derive comparison of toxicity of HMF towards larvae and adults. Adults showed an extrapolated LC_{50} nearly 20 times higher than those of larvae on d7, whereas on d22, LC_{50} of larvae was 1.3 times higher than that of adult ones (Tab. II). This might be a result of the mentioned longer exposure to HMF and the higher consumed overall-dose of caged adult workers.

Previous comparison of toxicity on larvae and adult honey bees has been very limited. The Apenet Project [2009] in Italy delivered results on toxicity of two pesticides, fipronil and the neonicotinoid clothianidin, on both larvae and adults. Astonishingly larvae showed a much higher resistance after 24h of acute intoxication than adult workers. As adults evinced a LD_{50} of about 4 ng for both substances, larvae showed a LD_{50} of 39 ng for fipronil and more than 3000 ng for clothianidin. The fact that both substances act on the nervous system of insects and larvae present a comparatively simple nervous system might be the reason, why these substances are less toxic to larvae.

Beekeepers should notice the danger of HMF contamination and purchase their supplemental food from reliable sources or, when manufacturing sugar syrup by themselves, be careful not to produce or store under conditions that favour the building of HMF. Using not sold, possibly several years old honey as supplemental food might be noisome, especially regarding results like those of ALabdeen Makawi [2009], where long term stored honey can develop HMF-concentrations up to more than 900 ppm. When kept between 15-25°C, honey stored for 28 months shows HMF concentrations below 60 ppm [Sancho et al., 1992].

Further research is needed to test more detailed concentrations ranging between 750 and 5000 ppm HMF to see when first significant differences in mortality occurs and hence determine a NOAEC (No Observed Adverse Effect Concentration) or LOAEC (Lowest Observed Adverse Effect Concentration), which should be found above 750 ppm HMF. Under natural conditions, honey stored within the hive will probably less likely develop a HMF concentration as high as the LC_{50} determined in our study. However, it is conceivable, that

supplemental food, acid-catalyzed and long-term stored under improper conditions, might reach a HMF- level that can cause lethal or sublethal damage in a dimension that is not yet known. Indeed, the impact of sublethal HMF intoxication is needed to be investigated, as, especially in combination with other stressors, it might reduce resistance against environmental influences, pesticides, parasites and diseases.

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