Detecting the influence of different potential stress factors on the behavior of the honeybee *Apis mellifera* using Radiofrequency Identification (RFID)

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III. General Summary

This study was conducted to determine the influence of different stress factors on the honeybee *Apis mellifera*. The investigation was motivated by previous experiments that suggested the existence of an unspecific defense mechanism causing a generalized change of flight behavior after the onset of different diseases. This mechanism is thought to impede the ability of flight bees to return to their respective colonies thereby removing the disease from the colony over time. During the last years, the existence of such a “suicidal behavior” was supported by further studies. Thus, an unnoticed, potentially highly effective defense mechanism of social insects was revealed whose spectrum of activity and physiological basics require further investigation. Suggesting that the reaction by the bees is unspecific to different diseases as well as to other potential stress factors, this study was designed to investigate the influence of pathogens, insecticides, and different brood rearing temperatures on different parameters like lifespan, foraging activity, and foraging trip duration of worker bees. The first and foremost aim was to develop a better experimental access for monitoring behavioral changes than by time and staff consuming observations, thereby providing a basis for more extensive investigations. For this purpose an automatic individual identification system base on radiofrequency identification (RFID) was used to determine the activity of individually labeled bees at the hive entrance. A further development made it possible to automatically detect bee activity at an artificial foraging source.
Experiments were conducted on the influence of infection with the endoparasitic microsporidium *Nosema sp.*. Bees were labeled with RFID transponders, artificially inoculated with *Nosema* spores, and monitored over their total lifespan. Infections with Nosema lead to a reduced lifespan, an earlier increase in activity, and at least in parts to an increased foraging trip duration. This stands in accordance with results by previous studies on Nosemosis and Varrosis (*Varroa destructor*) which reported longer homing flights of and increased activity for infected/infested bees.
A further experiment focused on the influence of different brood rearing temperatures during pupal development, a development stage where developing bees are very sensitive towards changes of the environmental temperature. The beginning of foraging duties, foraging activity, foraging trip duration, and longevity were recorded for bees reared at different temperatures which have been shown to be present in the

brood area of a honeybee colony (32°C-35°C). It was observed that bees reared at above-optimal 36°C were overly active and spent the most time outside of the hive compared to the other two tested groups of bees (32°C, 35°C). Bees reared at an optimal 35°C lived longer than the 36°C- and the 32°C-bees.

Due to the actuality of the matter an additional focus was on the effect of insecticides as stress factors. The use of a semi-field method allowed the detection of sub-lethal effects caused by several insecticidal active ingredients on the foraging behavior of honeybees. Several parameters of foraging behavior including foraging activity, foraging trip duration, flight duration to the feeder and back to the hive, and time interval between foraging trips were measurable by RFID registration. After oral administration of different doses of the neonicotinoid substances imidacloprid and clothianidin, and of the organophosphorous varroacide coumaphos, an accaricide used for the control of the parasitic mite *Varroa destructor*, it was possible to detect differences in the foraging behavior of the treated bees. While the effects induced by clothianidin and imidacloprid at dosed of ≥0.5ng/bee and ≥1.5ng/bee, respectively were mainly detectable during a three-hour period immediately after administration the impact of coumaphos ≥2µg could be monitored up to 48h after treatment. This difference might be attributed to the different mode of action of neonicotinoids, agonists of the insect nicotinic acetyl choline receptor (nAChR), and organophosphates, inhibitors of the acetyl choline esterase, as well as to different modes of detoxification. Furthermore, the results indicate that there could be different targets for imidacloprid and clothianidin on honeybee motorneurons due to prolonged flightdurations after administration of imidacloprid which did not occur after administration of clothianidin, and due to different symptoms after treatment with higher doses of both substances.

The reported results confirm that the RFID technology provides an effective approach to monitor the behavior of individual bees around the clock during their entire lifespan without time and staff consuming observations. With the experimental approaches presented in this study it was possible to test for an influence of different stress factors and to determine those effects by accurate time measurements. The results underline furthermore, that different stresses like diseases, insecticides, or thermal stress produce similar responses by increasing the activity, reducing lifespan, or extending foraging duration.

Allgemeine Zusammenfassung

Im Rahmen dieser Studie wurden Untersuchungen bezüglich der Auswirkungen von unterschiedlichen Belastungsfaktoren auf die Honigbiene *Apis mellifera* durchgeführt. Hintergrund waren Vermutungen, die nahe legten, dass Bienen auf Parasiten und Pathogene durch eine generalisierte Verhaltensänderung reagieren, die die Rückkehr der Bienen in das Volk behindert und damit Krankheit nach und nach aus dem Volk entfernt. Die Existenz eines solchen „suizidalen Verhaltens“ wurde zwischenzeitlich durch weitere Untersuchungen unterstützt. Hiermit wurde ein bislang unbeachteter und potentiell hochwirksamer Abwehrmechanismus sozialer Insekten gegen Pathogene und Parasiten aufgedeckt, dessen Wirkspektrum und physiologische Grundlagen noch erheblichen Aufklärungsbedarf haben. Es lag nun nahe, eine allgemeine und unspezifische Reaktion auf Stressfaktoren zu vermuten. In dieser Studie sollte daher der Einfluss von Pathogenen, Insektiziden sowie unterschiedlichen Brutaufzuchtbedingungen auf die Aktivität, das Flugverhalten und das Sammelverhalten messbar gemacht und untersucht werden. Im Vordergrund standen dabei Bemühungen, die Erfassung der Verhaltensänderungen experimentell zugänglicher zu machen als dies durch personalaufwändige Einzelbeobachtungen möglich ist, und hiermit verbesserte Voraussetzungen zu umfassenderen Untersuchungen zu schaffen. Hierfür wurde ein automatisches Identifikationssystem verwendet, das auf der Radiofrequenz-Identifikations (RFID) Technik basierte. Hiermit war es möglich, die Aktivität von individuell markierten Honigbienen am Stockeingang zu erfassen. Des Weiteren kam es zu einer Weiterentwicklung, die eine automatische Registrierung auch an einer künstlichen Futterquelle ermöglichte.

Es wurden Untersuchungen zum Einfluss des Befalls mit dem parasitischen Mikrosporidium *Nosema sp.* durchgeführt. Hierfür wurden Bienen mit einer durch *Nosema*-Sporen kontaminierten Futterlösung infiziert. Die Infektion führte zu einer Verringerung der Lebensspanne, einer Erhöhung der Flugaktivität sowie, zumindest in Teilen, zu einer verlängerten Dauer der Sammelflüge. Diese Ergebnisse stehen im Einklang mit denen aus früheren Studien zur Nosemose, aber auch zur Varrose (*Varroa destructor*), bei denen nachgewiesen werden konnte, dass infizierte Bienen länger für Rückflüge zum Stock benötigen und auch aktiver sind als Bienen, die nicht infiziert wurden.

Ein weiteres Experiment beschäftigte sich mit dem Einfluss von unterschiedlichen Brutaufzuchttemperaturen während des Puppenstadiums, einer Entwicklungsphase,

in der die sich entwickelnde Biene sehr empfindlich gegenüber Veränderungen in der Umgebungstemperatur ist. Bienen, die bei Temperaturen, wie sie im Brutbereich des Bienenvolkes vorkommen können (32-36°C), aufgezogen wurden, wurden nach ihrem Schlupf auf den Beginn ihrer Sammeltätigkeit, die Aktivität, die Dauer der Sammelflüge sowie die Lebensspanne untersucht. Hierbei konnte festgestellt werden, dass Bienen, die bei für den Brutbereich etwas zu hohen 36°C aufgezogen wurden, früher aktiv waren und eine höhere Aktivität sowie längere Aufenthalte außerhalb des Stocks aufwiesen als die beiden anderen getesteten Temperaturgruppen (32°C, 35°C). Bienen, die bei für das Brutnest optimalen 35°C aufgezogen wurden, erreichten das höchste Durchschnittsalter, gefolgt von den 36°C und den 32°C Bienen.

Desweiteren wurden, auf Grund der derzeitigen Aktualität, Untersuchungen bezüglich des Einflusses von Insektiziden als Stressfaktoren durchgeführt. Die Nutzung einer Semi-Freilandmethode erlaubte es, nicht tödliche (subletale) Effekte unterschiedlicher insektizider Wirkstoffe auf das Sammelverhalten von Flugbienen zu erfassen. Hierbei wurden verschiedene Parameter des Sammelverhaltens mittels RFID-Messungen untersucht. Es war möglich genaue Messungen bezüglich Besuchshäufigkeit an der Futterquelle sowie Hin- und Rückflug und Aufenthalt an der Futterquelle und des Weiteren den Aufenthalt im Stock zwischen Sammelflügen durchzuführen. Nach oraler Verabreichung verschiedener Dosierungen der Substanzen Imidacloprid, Clothianidin (beide gehören zur Substanzklasse der Neonicotinoide) und Coumaphos (gehört zur Substanzklasse der Organophosphate) ermöglichte es diese Methode Abweichungen im Sammelverhalten der behandelten Bienen zu registrieren. Nach mündlicher Verabreichung von verschiedenen Dosierungen der Neonicotinoide Imidacloprid und Clothianidin sowie des Organophosphats Coumaphos, ein Akarizid zur Bekämpfung der parasitischen Milbe *Varroa destructor,* war es möglich Veränderungen im Sammelverhalten der behandelten Bienen festzustellen. Während die Auswirkungen, die durch Clothianidin und Imidacloprid, ab 0,5ng/Biene bzw. 1,5ng/Biene, ausgelöst wurden, hauptsächlich während der ersten drei Stunden unmittelbar nach der Behandlung zu beobachten waren, ließen sich die Effekte von Coumaphos (≥2µg/Biene) noch bis zu 48 Stunden nach Verabreichung nachweisen. Dieser Unterschied könnte sich mit der unterschiedlichen Wirkweise der Neonicotionoide, Agonisten insektischen nikotinischen Acetylcholin Rezeptors (nAChR), und der Organophosphate, Inhibitoren der Acetylcholin Esterase, sowie möglicher Unterschiede in der

Detoxifikation der beiden Substanzen erklären lassen. Verlängerte Flugdauern nach Behandlung mit Imidacloprid, die bei der Behandlung mit Clothianidin nicht auftraten, sowie unterschiedliche Symptome nach Verabreichung höherer Dosierungen der beiden Substanzen ermöglichen Vermutungen über unterschiedliche Typen von Zielrezeptoren für Imidacloprid und Clothianidin auf Motoneuronen von Honigbienen.

Die erzielten Ergebnisse bestätigen die Vermutung, dass es sich bei der RFID-Technik um eine Methode handelt, mit der es möglich ist, ohne aufwendige und zeitintensive Beobachtungen das Verhalten individueller Bienen rund um die Uhr über ihre gesamte Lebensdauer zu überwachen. Mit den verschiedenen in dieser Studie erarbeiteten experimentellen Ansätzen war es möglich, die Auswirkungen von unterschiedlichen Belastungsfaktoren, zu denen Pathogene, Insektizide und andere Umwelteinflüsse wie z.B. Temperatur gehören, durch sekundengenau erfasste Messdaten sichtbar zu machen. Die Ergebnisse unterstreichen ebenfalls, dass unterschiedliche Stressfaktoren wie Krankheiten, Insektizide oder Temperaturbelastungen sich ähnlich auswirken, indem sie die Aktivität der Bienen außerhalb des Stocks erhöhen, zu verkürzter Lebensdauer führen oder aber die Sammeldauer verlängern.

IV. General Introduction

Honeybees have been subject of many studies over the past centuries. They are well known for their production of honey, and also play an important role, together with other pollinating insects like feral- and bumblebees, in the pollination of crops grown for human consumption (A.-M. Klein et al. 2007). The development of so-called observation hives made it possible to observe and identify complex honeybee behaviors as well as their division of labor within the colony without disturbing them as was the case when opening a hive box. This allowed detailed insights into honeybee communication, broodcare, and many other tasks necessary for the hive community. While achievements like von Frisch's encoding of the honeybee dance language (von Frisch 1967), or thermo graphic images of bees heating up the brood area to a temperature of around 35°C (Bujok et al. 2002; Kleinhenz et al. 2003), enabled detailed determination of different in-hive behaviors, a far more difficult challenge is the observation of the out-of-hive behavior of honeybees, and here especially of the flight behavior. Classical visual and/or camera observation methods allowed tracking of bee departures and arrivals at the hive entrance. Nevertheless, these observation-studies on individual flight behavior are severely confined since they are time-consuming and require quite some human resources for the actual observations or the screening of video materials, putting limitations on the numbers of individuals investigated and the time for analyzing them.
The introduction of a path-tracking system using harmonic radar (Riley et al. 1996) has enabled detailed insights into the flight routes of honeybees (Capaldi et al. 2000; Menzel et al. 2005), but the high operating costs of the required devices, the need to remove the harmonic transponders after each flight, and the special terrain requirements for this technique allow only restricted use. The application of automatic data collection by radio frequency identification technology (RFID) is of considerable advantage compared to the classical methods (Sumner et al. 2007) and could be extremely useful in the studies of insect behavior (Streit et al. 2003; Decourtye et al. 2011; Pahl et al. 2011). Attaching RFID tags on the thorax of bees enables simultaneous tracking and identification of numerous individuals at the colony entrance, where scanner devices are positioned above the entrance, supplying the power to the passive RFID tag which then sends the identification information back to the scanner.

It was already shown, that bees, which are challenged by pathogens like e.g. the parasitic mite *Varroa destructor* or the endoparasite *Nosema* sp., show alteration in their flight behavior resulting in longer homing flights or not returning at all when released in the perimeter of the hive (Kralj and Fuchs 2006, Kralj and Fuchs 2009). Furthermore, it was shown that a higher proportion of infected bees did not return to the colony after leaving it for foraging trips. The authors believe that this can be understood as an adaptive defense mechanism leading to a reduction of the pathogen load through the inability of infected bees to return to their colony due to a possibly impaired orientation. Such a “suicide hypothesis” was already discussed by Smith-Trail (Smith-Trail 1980) and was recently shown in two ant species *Forelius pusillus* and *Temnothorax unifasciatus* (Heinze and Walter 2010; Tofilski et al. 2008). The idea based on this study is that this described mechanism is not a specific reaction to disease but a more or less general defense mechanism against honeybee stresses which in addition include sublethal amounts of chemical substances e.g. pesticides, as well as environmental influences like nutrition and temperature. Most recently, a study was published by Rueppell and colleagues (Rueppell et al. 2010) that observed reduced lifespan after prolonged narcosis with CO_2 and the cytostatic drug hydroxyurea and thereby further supports the idea of an unspecific defense mechanism against stresses.

This study is split up in three parts and had the intention to test the applicability and reliability of the RFID method by independently examining the influence of potential stress factors on longevity, flight-, and foraging behavior of honeybees.

In the first part of this study the animals were monitored for effects caused by inoculation with the microsporidian endoparasite *Nosema* sp. which is already shown to affect the lifespan of infected bees (Borchert 1928; Maurizio 1946; Beutler, Opfinger, and Wahl 1949) as well as their flight behavior (Kralj and Fuchs 2009). Instead of these classical observation methods on individual bees taken from a colony, the RFID method provides lifelong automatic monitoring of colony entrance passages from which a much broader set of parameters can be inferred, including first and last passage of bees at the entrance which give an estimate of active forager life, but also the number of flights per bee, the duration of these flights, and losses of bees from the colony referring to the total lifespan. Furthermore, it is examined whether the data set obtained by the RFID method would support the adaptive suicide hypothesis (Smith-Trail 1980; Rueppell, Hayworth, and Ross 2010)

also investigated in the other social insect species (Heinze and Walter 2010; Tofilski et al. 2008) with regard to the influence of Nosema sp. on honeybee foragers.

The second part of this study focuses on the influence of an important factor in the development of the honeybee, that is, the temperature during brood rearing. It was shown before that bees need a relatively narrow temperature range between 32°C and 36°C (Himmer 1932; Jay 1963; Seeley 1985; Himmer 1927) especially during the sensitive pupal development stage (Koeniger 1978) for normal development. Longer periods of temperatures above or below these thresholds were found to lead to increased brood mortality and malformations of the newly hatched imagines (Himmer 1927; Weiss 1962; Koeniger 1978; Groh, Tautz, and Rössler 2004), while deviations within this range have been shown to change the behavior and neural physiology in the animals affected (Tautz et al. 2003; Groh, Tautz, and Rössler 2004; Bock 2005; Becher, Scharpenberg, and Moritz 2009). To assess if these results were reproducible with RFID monitoring, previously sealed brood combs were removed from their colonies and incubated at three different temperatures of 32°C, 35°C, and 36°C. Immediately after hatching these bees were labeled with RFID transponders and were introduced into a foster registration colony for lifelong monitoring of activity, flight durations, and lifespan.

The third part of this study was to use a method, also independently developed by Decourtye and colleagues (Decourtye et al. 2011) to detect sublethal effects of different insecticidal compounds on the foraging behavior of honeybees. The conflict between farmers and beekeepers concerning the use of insecticides in agriculture on one hand and the significance of honeybees and other insects for crop pollination (A.-M. Klein et al. 2007) on the other, have attracted increasing scientific and public attention, due to increasing honeybee colony losses and declining pollinator populations. Reliable and efficient methods to be used in field tests, assessing the less obvious sublethal effect of insecticides, which are already known to induce behavioral changes in honeybees in bioassay laboratory tests (K.S. Taylor, Waller, and Crowder 1987; Stone, Abramson, and Price 1997; Lambin et al. 2001; Weick and Thorn 2002; Decourtye, Lacassie, and Pham-Delègue 2003; Decourtye et al. 2004; A. K. El Hassani, M. Dacher, et al. 2005; Thompson and Maus 2007; A. K. El Hassani et al. 2008) are still lacking. Since low doses of insecticides are known to disrupt learning and retention processes necessary for the correct association of olfactory or visual stimuli offered by a plant with a reward in form of nectar and pollen as shown in the laboratory, they also might cause visually non-observable effects on

motoractivity and/or orientation ability, leading to reduced foraging activity or prolonged foraging flights or no flights at all. While Decourtye and colleagues focused on the effects of the phenylpyrazol fipronil under tunnel conditions. This study on the one hand observed the effects of two systemic substances commonly used in seed dressings: the neonicotinoid insecticides imidacloprid and clothianidin. Both of these substances should only appear in low amounts in bee relevant products e.g. nectar and pollen (Schmuck et al. 2001; Schmuck and Keppler 2003). On the other hand I was interested in testing for effects of insecticidal substances that are directly applied into the hive. This especially is the case for the varrocides, acaricide substances used for the control of the parasitic mite *Varroa destructor*. Therefore, the organophosphate Coumaphos, active ingredient in different varroa control products, was chosen for investigation of its effect on foraging behavior of treated bees. Similar to Decourtye (Decourtye et al. 2011) experimental design not only allowed monitoring of RFID labeled honeybees at the hive entrance but also at an artificial foraging source, to which they were trained. Thus, detailed information about different phases of foraging as well as changes in behavior of honeybees, treated orally with low doses of the two neonicotinoid substances (imidacloprid, clothianidin), and the varroacide (coumaphos) was obtained.

V. General Methods

V. 1. General Description of the Radiofrequency Identification (RFID) System

RFID-systems were initially developed for tagging and identification of stored goods. They are non-contact identification systems normally consisting of two components. The transponder (tag), attached to the object that needs to be identified, and the reading device (scanner) needed to retrieve the information stored on the transponder (Finkenzeller 2002). The tag is the data storage medium of the RFID-system and usually lacks its own power supply, allowing a greater level of miniaturization. The energy needed to retrieve the information stored on the tag is provided by the scanner via inductive coupling. As soon as the tag enters the operating distance of the scanner it will be activated, enabling the transfer of the identity data to the scanner (Finkenzeller 2002).

The majority of RFID tags consist of a spatially separated transponder coil (antenna) and transponder chip (hybrid technology, (Finkenzeller 2002). This design limits the level of miniaturization. Thus, a different tag design was used in this study, where the antenna is planarly coiled on the surface of the chip (Coil-on-Chip, (Jurisch 2001; Finkenzeller 2002). This results in dimensions (0.8 or 1.6mm^3 and 2 or 4mg, respectively, **Fig 1**) which are appropriate for the use with honeybees, as first shown by Streit and colleagues (Streit et al. 2003).

Figure 1: left: Bees tagged with transponders of the new generation (1.6mm^3) feeding from an artificial feeder. right: Bees tagged with an old-generation transponder (0.8mm^3) at the hive entrance.

The RFID equipment (tags, scanners, software) for the different studies described in the following study was provided by microsensys GmbH, Erfurt, Germany. The tag belonged to the mic 3-series (mic 3-64D) with a 64 bit read-only (RO) memory containing the ID-number. It works at a frequency of 13.56 MHz and the reading distance is limited to 2 – 4mm. The reader model used was the 2k6 HEAD (memory: 512 kByte, memory capacity: ~33700 datasets, manufacturer's instructions). A special characteristic of these scanners is their direction sensitivity. Two separate, cascaded reader-antennas gave information on the direction of movement of an RFID-tagged honeybee allowing not only statements about the activity of the bee but also about the time the bee spent outside and inside the colony, respectively. To retrieve collected data from the readers, they are equipped with a serial interface to establish a connection to a computer. A USB to Serial (ATEN USB to SERIAL ADAPTER, chipset PL 2303) adapter was used for the data transfer.

As already mentioned, this RFID system provides a limited reading distance. Thus, it was a major requirement that the tag was aligned in the correct position (facing upward) for the scanner to register it effectively. Therefore, custom-made bee tunnels were positioned in front of the hive entrance, above which the scanners were mounted. The bee tunnel-model used by Streit and colleagues was modified for this study to ensure the best possible registration accuracy (**Fig. 2A**). The new cross-section of the tunnel took into consideration that bees prefer to extend their legs sideways while running along any surface, resulting in the wider lower cross-section part while the upper part was slim and arched to fit the thorax with the attached tag. Two tunnels were positioned in front of every colony (**Fig.2B**), avoiding overly crowding in times of traffic, each tunnel being monitored by a separate scanner.

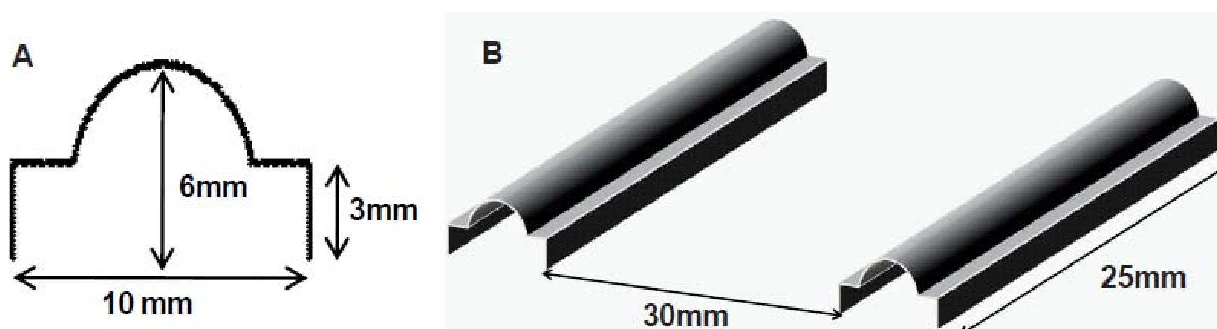

Figure 2: Cross section of the bee tunnels and their alignment in front of every registration hive. (A) The lower wider region of the cross section should encourage the bees to pass the tunnel with the dorsal side facing upward allowing an effective registration. (B)Two tunnels in front of every hive reduced jamming in times of high traffic.

Another modification made in comparison to Streit et al. regards the Plexiglas® base structure on which the tunnels are installed (**Fig. 3A**). The width of the structure from side wall to side wall chosen by Streit resulted in a direct connection of each tunnel to the respective side wall. Observations of bees passing the tunnels on this base revealed that particularly bees leaving the colony, running along the Plexiglas® side-walls, entered the tunnels sideways due to the close distance to the wall, resulting in a missed registration of the tag. Thus, the distance between tunnel entrance and side wall was extended to 8mm on each side (**Fig. 3B**) by building wider Plexiglas® bases (**Fig. 3A**). This and the framing of the tunnel entrances with wire mesh on both ends helped to increase accuracy of registration, though of course it could never be completely avoided that few bees passed the tunnel in a wrong fashion.

A)

B)

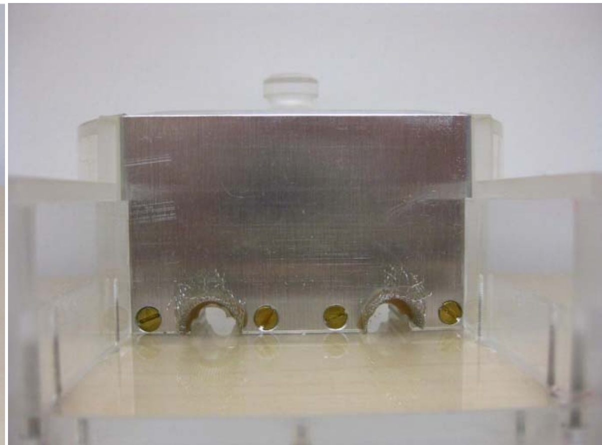

Figure 3: (A) Plexiglas® base (left: new model, right: old model) which is positioned in front of the hive and serves as a support for the double tunnel (B).

V. 2. The process of labeling bees with RFID tags

If not stated otherwise in the following chapters the RFID tags were attached in the following fashion. With a few exceptions the glue used was a non-toxic shellac-based, viscous adhesive (glue was provided in pack with numbered multicolored discs used for queen marking, Carl-Fritz-Imkereifachhandel, Mellrichstadt, Germany). Prior to tagging, a bee was introduced into a so-called queen marking tube (Carl-Fritz-Imkereifachhandel, Mellrichstadt, Germany, see also **Fig. 6**, Chapter V. 3.2.) These plastic tubes had a diameter of 30 mm and a length of approx. 75 mm. An elastic mesh closed off one end of the tube. A plunger padded with soft foam plastic was used to gently push and trap the bee against the mesh, exposing the upper part of the thorax through a mesh hole.

The most effective gluing procedure, guaranteeing the best adhesive performance, without using possibly harmful superglue (cyano-acrylite based) turned out to be the following:

A thin layer of liquid shellac (Lumberjack Schellack-Streichlack-natur, Alfred Clouth Lackfabrik GmbH&Co., Offenbach, Germany) was applied with a fine brush to ground coat the surface of the thorax, removing hair and wax residues possibly impairing the adhesion. A series of seven bees was treated this way consecutively, to ensure that the ground coating of the first bee had sufficiently dried following the application on the 7th bee. Subsequently, a drop of the viscous shellac-based adhesive was applied on the coated thorax immediately followed by the tag which was gently pressed on the glue. This step was done in rapid succession for every single one of the seven prepared bees. The procedure was then repeated with the next seven bees etc..

The adhesive needed to dry for a period of about 15 - 20 minutes. During this time, the marking tubes were stored in a specially-crafted rack. After the drying period was completed the plunger was removed from the mesh enabling the now labeled bee to move freely inside the marking tube. Before the release, it was observed if the bee could remove the tag on its own. In this case the bee was discarded and was replaced by a substitute. Otherwise, the tagged bees were transferred into a stainless-steel cage (see also **Fig. 5**, Chapter V. 3.2.) where they were provided with sugar dough (a mixture of sugar and honey with a ratio of 10 : 3.5, w/w). Depending on the experimental approach, the bees from the cage were released either outside or inside the colony after the last bee had been tagged.

VI. Monitoring disease-induced behavioral changes in forager honeybees Apis mellifera at the colony entrance.

VI. 1. Summary

Parasites are known to cause a broad range of behavioral changes in their hosts either benefitting or penalizing the latter. In honey bees, the parasitic mite *Varroa destructor* and the microsporidia Nosema sp. alter the foragers' flight behavior to the effect that the likelihood with which they return to the colony is decreased. In order to test if the RFID method, with less expenditure, could produce similar results already reported by studies using classical observation methods, bees were artificially inoculated with *Nosema* spores and monitored for their activity, foraging duration and longevity. We found reduced longevity (14% and 62%), earlier activity, and a significant negative relationship between activity and longevity of the inoculated groups in consecutive experimental years. Furthermore, in one year a significantly prolonged foraging duration was observed for the inoculated group in comparison to the non-inoculated group. These findings corroborate results obtained in previous studies regarding reduced longevity and flight behavior. They also confirm the presumption that early activity in infected bees leads to reduced lifespan in the end which was nearly impossible with classical observation methods.

VI. 2. Introduction

VI. 2.1. Nosemosis

Nosemosis in honeybees is a disease that predominantly infects the epithelium of the ventriculum (mid-gut). Two species of these endoparasitic Microsporidia are known to infect honeybees: *Nosema apis* and *Nosema ceranae*, the latter of which was first reported from the Asian honeybee *Apis* ceranae by Fries in 1996 (Fries et al. 1996). It was recently found to infect the European race of *Apis mellifera* as well (Higes et al. 2006; Ingemar Fries et al. 2006; Klee et al. 2007). The following part will focus on the biology *Nosema apis* and its effect on physiology and behavior of the infected honeybees since they have already been well described, while it is likely that they are similar in *Nosema ceranae*. Recent studies by Forsberg and Fries have shown that there is no significant difference in the mortality causes by both Nosema species (Forsgren and Fries 2010), and that virulence of Nosema ceranae seems to differ with regard to different environmental conditions (Fries 2010).
Nosema can virtually be found all around the world with the exception of some parts of Africa, the Middle East, or the Malay Archipelago (Bradbear 1988).

VI. 2.1.1 Infection, Multiplication, and Spread

Infection occurs through oral ingestion of Nosema spores, the only state in which the parasite is viable outside the mid gut. The spores are oval shaped with a mean size of 6 x 3µm and contain the primary form of the parasite. The presence of spores is usually diagnosed by microscopic examination. There are several transmission routes. The most common infection route is through contact with feces defecated in droplets inside the hive. Usually worker bees defecate outside of the hive, but factors like inclement weather in summer (Borchert 1948) and a tightly filled rectum especially at the end of the winter period (heightened metabolism due to the start of brood rearing, Nitschmann 1957) may lead to defecation of bees on the combs. The spores leave their host together with the feces just to be taken up by other bees trying to do cleaning duties inside the hive. While Nosema infection is likely to be accompanied by dysentery accelerating the spreading of the disease, it was shown

by Bailey (L. Bailey 1967) that Nosema is not the prime cause for dysentery. Other potential ways of spreading the disease are trophallaxis (transfer contaminated food to other hive mates), drifting of infected bees into uninfected colonies, robbery of infected, weakened colonies, and poor beekeeping practice using contaminated combs for new bee nuclei (Zander and Böttcher 1984).
After the ingestion the spores quickly pass (less than 10 minutes, Kellner & Jacobs 1978) through the Proventriculus (proventricular valve) into the mid gut, where most of the digestion and absorption takes place (L. Bailey 1952; Dietz 1969). Once there, the spore everts its around 400µm long, hollow polar filament (Lom and Vavra 1963) and injects the germ into the epithelial cells, if close enough (Kramer 1960; Morgenthaler 1963; Fries 1988; Weidner et al. 1984). In the cytoplasm of these cells, the parasite starts developing and multiplying (asexually). At the end of the development between 48 and 60h later (Fries 1988) new spores are formed inside the cell. When these epithelial cells are sloughed off into the intestinal lumen making room for new ones, the released spores either germinate, infecting new cells (autoinfection), or move to the rectum with the other gut contents and are excreted with the feces (Leslie Bailey and Ball 1991; Ritter 1996). Gross and Ruttner (Gross and Ruttner 1970) found that after 8-10 days after infection the parasite has spread throughout the entire intestinal wall.

VI. 2.1.2. Influence of Nosemosis on physiology and behavior

Many effects on honeybee physiology and behavior have been well reported in connection with nosema infection.
The sloughing off of epithelial cells, a normal process in honeybees, happens more rapidly in infected bees (Trappmann 1926). This leads to a continuous degeneration of the midgut and its metabolic function, disrupting the secretion of digestion enzymes needed to degrade proteins (Beutler, Opfinger, and Wahl 1949; Ritter 1996; Malone and Gatehouse 1998). Lotmar (Lotmar 1951) observed an increased weight and water content of the rectum which explains the increased need for defecation. Wang and Moeller (D. I. Wang and Moeller 1969) found less developed hypopharyngeal glands, which are needed to provide the larvae with proteins, lipids, and vitamins and Liu (Liu 1990) reported less concentrated gland secretions in

Nosema infected honeybees. This would explain findings that eggs of severely infected colonies fail to develop into larvae more often than in non-infected colonies (Hassanein 1951), and that infected bees switch from nursing to other tasks more rapidly (D.I. Wang and Moeller 1970). Hassanein (Hassanein 1953) found that infected bees start foraging earlier with the time of the in-hive duties being reduced from 17-18 days (non infected) to 8-11 days. In laboratory- and hive experiments a reduced lifespan was reported for Nosema infected bees in several studies. Maurizio (Maurizio 1946) who used young summerbees and winterbees of unknown age in his cage experiments found a reduced lifespan by 18% (five days) and 36.5% (13.2 days), respectively. Beutler and colleagues (Beutler, Opfinger, and Wahl 1949) who kept young summerbees in a cage with additional pollen supply reported that the mean lifespan was reduced by 24.3% (8.5 days). Borchert (Borchert 1928) introduced 700 uninfected and 700 infected bees into each of two different colonies. Only 23.8% and 21.3% of the infected populations were found compared to 50.9% and 73.4% of the control populations after about three weeks in both colonies, respectively.

In addition to the precocious switch from in-hive to foraging tasks, it was also recently shown that Nosema infection led to alterations of flight and homing behavior, and orientation. Kralj and Fuchs (Kralj and Fuchs 2009) reported that infected bees need 2.1 times longer to return to the hive compared to uninfected bees when released outside of the hive. Furthermore, they showed that infected bees failed to return to the hive more often (2.7 times) than non-infected bees and that infected bees approached a dummy-hive entrance (positioned next to the original entrance) more frequently (69.3%) than the uninfected bees (32.4%).

Kralj and Fuchs (Kralj and Fuchs 2006) found similar results for bees infested with the parasitic mite *Varroa destructor* which they interpreted as self sacrifice of diseased bees for the benefit of the colony. Such a suicidal mechanism (Smith-Trail 1980) that was also investigated in other social insects such as the ant species` *Forelius pusillus* and *Temnothorax unifasciatus* (Heinze and Walter 2010; Tofilski et al. 2008) could help to reduce the pathogen load of the colony.

By using RFID-transponders for automatic registration at the hive entrance, I want to provide additional information about the development of Nosema infected bees, and prove that it is a fitting method for monitoring of the effects of Nosema infection in

great detail over the complete honeybee lifespan without protracted visual observation.

VI. 3. Material and Methods

The studies on the effect of the *Nosema* disease were conducted at the facilities of the Institut für Bienenkunde in Oberursel. Lifespan, activity at the hive entrance, and time period spent outside the hive were observed in repeated measurements during the years 2008 and 2009.

VI. 3.1. Bee Material

All broodcombs and nuclei bee hives needed for this experimental series (one comb per experiment) were taken from colonies housing an *Apis mellifera carnica* breeder line, belonging to the "Institut für Bienenkunde".

VI. 3.2. Experimental Procedure

Figure 4: Color mark on worker bee.

The timeline (**Fig. 5**) for each of the three experiments conducted was as follows. A broodcomb with mature brood cells was removed from the colony and kept overnight in an Incubator (Rumed, Rubarth Apparate GmbH, Laatzen, Germany) at a temperature of 35°C and a humidity of 65-75%. On the following morning, all newly hatched bees were collected from the comb and color marked (Edding® 750 paint marker, edding International GmbH, Ahrensburg, Germany) dorsally on the thorax (**Fig. 4**). Subsequent to the color marking the young bees were introduced into a nucleus bee hive (Mini-Plus, Bienenzuchtbedarf Heinrich Holtermann GmbH & Co KG, Brockel, Germany) which served as registration hive. After four days, the color marked bees were collected from the combs of the registration hive. A number of sixty bees were labeled with RFID-tags before being divided into two groups. Each group was confined in a cage. Both groups were bulk fed with honey solution (33% weight/volume). The control group (Nosema-) received pure solution, the Nosema group (Nosema+) received honey solution contaminated with *Nosema* spores (5 x 10^6 spores/milliliter). For the subsequent determination of a successful *Nosema* infection an additional positive- and negative control were introduced. Forty additional bees from the group collected of the combs of the registration colony were added to each cage. Instead of with an

RFID-Tag, these bees were labeled with an additional, different color-marking: blue for Nosema- and red for Nosema+ group. After the 70 bees in each cage had emptied the offered honey solution they were reintroduced into the registration colony with a delay of 15-30 minutes between the cages to avoid trophalaxis between inoculated and non-inoculated bees. Twelve days later the additionally color marked positive and negative controls were collected from the hive and examined for *Nosema* infection. The RFID-labeled bees remained in the colony and were not disturbed again.

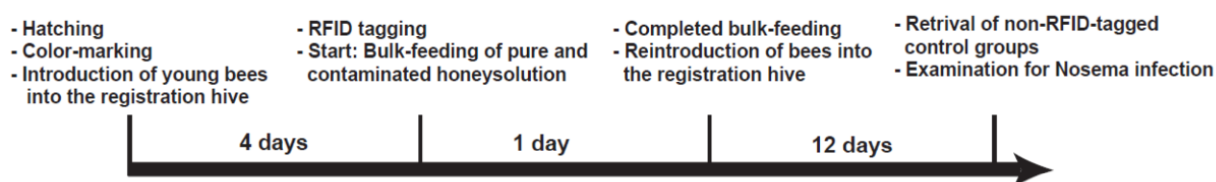

Figure 5: Timeline of the preparations for the *Nosema*-experiments. The period of time between two sections refers to the time interval between these sections and is not an added up value.

VI. 3.2.1. Preparation of the *Nosema* solution and examination for *Nosema* infection

The examination for *Nosema* infection was conducted in a Neubauer improved counting chamber. Bees for this experiment were infected with fresh spores obtained from the midgut of previously infected bees. Therefore, the digestive tract of the latter bees was excised with a forceps, followed by an isolation and maceration of the midgut in 0.1ml H_2O. A droplet of this suspension was placed on the counting chamber and was investigated for spores under a light microscope (400x magnification). The total number of spores in the midgut was assessed by cell counting in three 0.0025mm^2 squares (depth: 0.1mm) in the center of the counting chamber, and subsequently the *Nosema* solution diluted with honey water to a concentration of 5 x 10^6 spores / milliliter. Part of the solution was used for identification of the *Nosema* species using PCR technique (Cox-Foster et al. 2007). After the retrieval of the additional positive and negative controls for infection, the bees were deeply frozen. The midgut of each bee was excised and crushed in 0.1ml of H_2O and checked for the presence of *Nosema* spores. The criterion for *Nosema sp.* infection was the presence of spores in every microscopic observation field.

VI. 3.2.2. Data analysis

The transponders for the Nosema+ (inoculated) and the Nosema- (non-inoculated) groups were read in and saved shortly before the labeling process began (see also Chapter IV. 2, p. 15). Four main parameters were analyzed: I) The lifespan of every tagged bee which was defined as the period from hatching until the day of the last registration. II) Decline of the experimental populations over time. III) The activity at the hive entrance, where each movement either directed out- or inward was counted as one activity. IV) The time period spent outside of the hive and its development with increasing age of the bees. Here, a filter was used to search only for times that were longer than 30 seconds This helped to erase many short-duration stays outside the hive which possibly occur during guard duties and quick in and out movements

The lifespan in days for both groups was determined and then tested for normal distribution with Shapiro-Wilk-Test. If the data was normally distributed the mean lifespan of the different groups was compared by oneway ANOVA. If the result was found to be significant a pair wise analysis was conducted by using the Bonferroni-post-hoc-test. If the data was found to be not normally distributed a non-parametrical Mann-Whitney-U-Test was conducted. Activity per bee and the median time that each bee spent outside the hive were compared in a similar fashion for every week of their lives. In addition to the average lifespan, the decrease of the experimental populations over time was observed by Kaplan-Meier survival analysis. The pairwise comparison of the survival curves was done with Breslow –Test (SPSS Statistics 17).

VI. 4. Results

The examination of the additional positive control revealed that of the 67 bees collected from the hive during the three experiments, 57 were infected with *Nosema* spores (85.1%, **Tab. 1**). *Nosema* spores were detected in the intestines of all thirteen bees collected in 2009 (20.08.2009). They were classified as high (n=3), medium (n=7), and low infected (n=3) according to Ritter (1996). Results from the previous year showed an infection success of the positive control of 76% (high: n=6; low infected: n=13) and 86.2% in the first (10.07.2008) and second experiment (06.08.2008), respectively. From the total number of 73 bees belonging to the negative control group, 70 (96%) were found to be *Nosema* free (10.07.2008: 96.3, 06.08.2008: 100%, 20.08.2009: 90.5%). The samples of the infective solution in both years used for species analysis contained *N. ceranae* only.

Table 1: Examination of the additional control groups for *Nosema* infection.

	2008		2009	
	10. July	06. Aug	20. Aug	Total
Positive Control (N+)	N(bees)	N(bees)	N(bees)	N(bees)
Infected	19	25	13	57
non infected	6	4	0	10
Total	25	29	13	67
Negative Control (N-)	N(bees)	N(bees)	N(bees)	N(bees)
Infected	1	0	2	3
non infected	26	25	19	70
Total	27	25	21	73

Due to lack of data collected during the experiment from the 06.08.2008 a detailed analysis was not feasible. Only 1300 data sets were saved on the scanners, a small amount compared to the > 6900 data sets collected during the experimental periods of the other two experiments.

Of the 60 RFID-tagged bees inserted into the colony in 2008, a total 45 [N (Nosema-) = 19, N (Nosema+)= 26] were registered at the hive entrance. In the following year 59 of 60 bees [N (Nosema-)= 30, N (Nosema+)= 29] were registered again after being introduced into the registration hive.

VI. 4.1. Monitoring of Lifespan

During both experiments conducted in 2008 and 2009, a reduced lifespan was observed for bees that had been inoculated with *Nosema* spores. In 2008 the Nosema+ bees were registered over a median of 46.5 days (**Fig. 6, left)**. Their lifespan was reduced by 14% compared to the Nosema- bees that were registered over a median of 54 days. Nevertheless, this difference was not verified by statistical analysis using the Mann-Whitney-U-Test ($p \leq 0.093$). In contrast, a significant difference concerning the lifespan of both groups was found in the following year ($p \leq 0.001$) (**Fig. 6, right**). While the Nosema- bees were registered over a median 50.5 days in 2009, the median lifespan of the Nosema+ bees (19 days) was reduced by 62.4%.

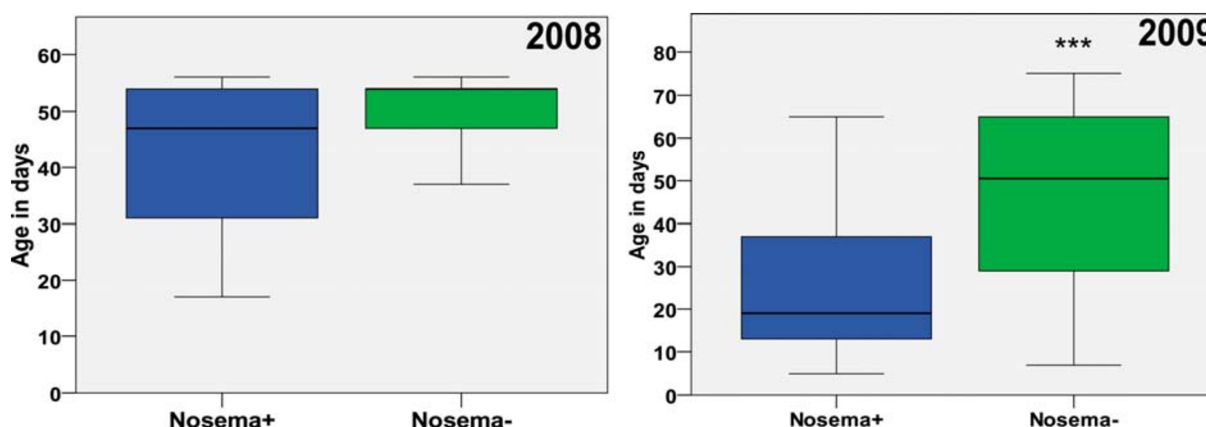

Figure 6: Effect of *Nosema*-infection on honeybee lifespan. Plotted is the lifespan of every bee in days during the experiment in 2008 (left) and 2009 (right). In the first experiment a non-significant tendency towards a shorter lifespan for artificially inoculated bees was observed. In the following year, bees inoculated with *Nosema* spores disappeared significantly earlier compared to the non-infected control group. (● = outlier, single asterisk = extreme outlier, multiple horizontally aligned asterisks = significance level)

The maximum age reached by a Nosema+ and a Nosema- bee in 2008 was 56 days. A registration time of 50 days or longer was recorded for eight individuals of the N- group, and for ten individuals of the N+ group. In the following year, maximum registration time for both groups was 75 days. Sixteen individuals of the N- group were recorded for 50 days and longer compared to four individuals of the N+ group. The onset of the disease was expected to be about ten days after inoculation. This would match an age of approx. 15 days. No obvious decrease of the N+ population was found at that time in 2008 compared to the non-infected control group (**Fig. 7 A**), but rather ten days later. At that point, the survival curves start to differ from one another with the N+ curve dropping more quickly than the N- curve. Nevertheless, this difference was not found to be statistically significant ($p = 0.278$, Breslow -Test).

In 2009 a significant decrease of the N+ population was observed around day 15, the presumed time of the disease onset (**Tab. 2**, **Fig. 7 B**).

Table 2: Result of Breslow Test used to compare the survival curves of both groups.

		Year	Chi^2	p-value
Nosema-	Nosema+	2008	1.178	0.278
		2009	11.858	0.001

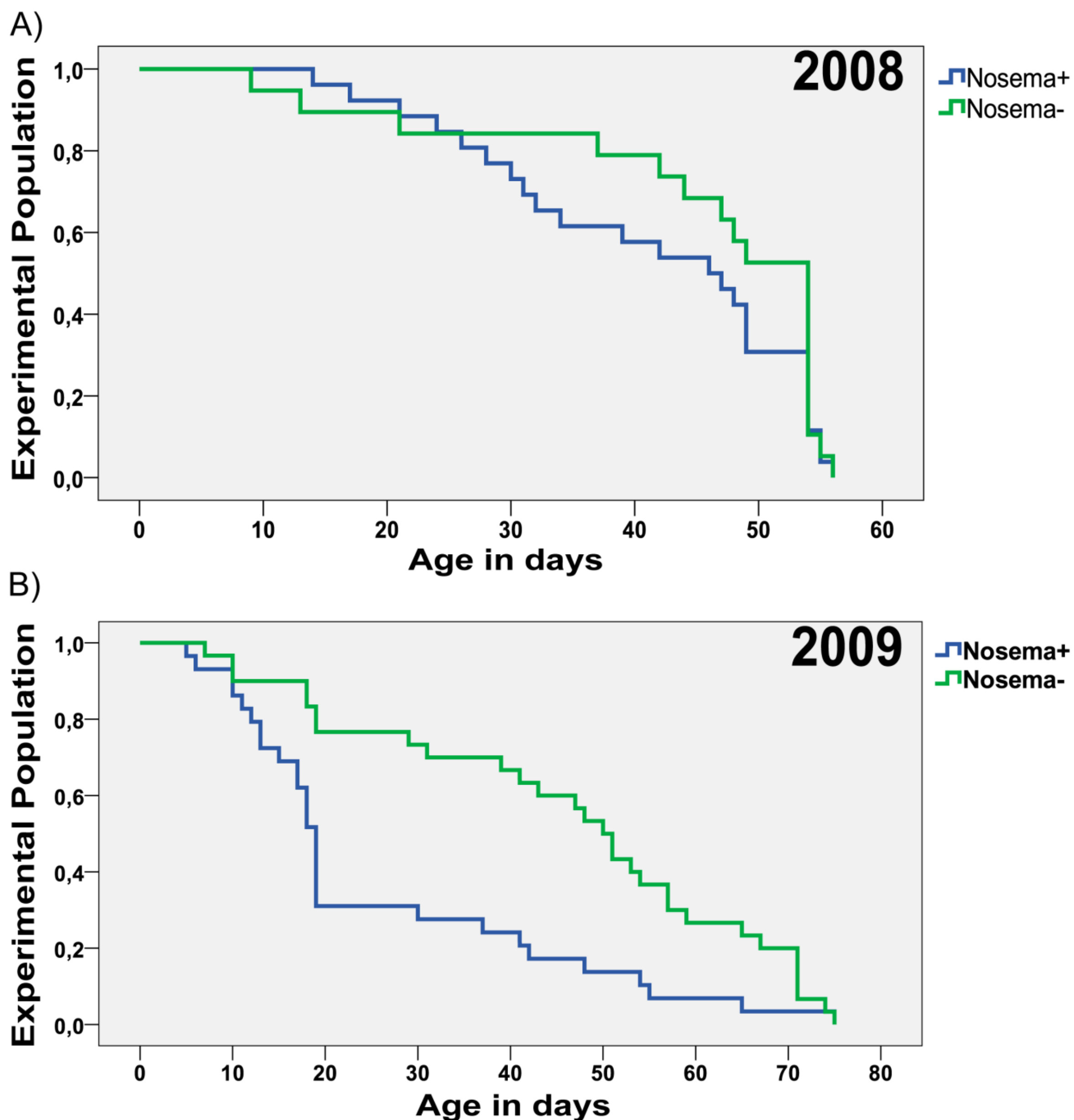

Figure 7: Decrease of the experimental populations during the *Nosema* experiments.
In 2008 (A) a pronounced, though non-significant, decrease of the population of *Nosema*-infected bees can be observed from day 25 forward. Half of the population has disappeared after day 47 and 54 for the infected and non-infected group, respectively.
In 2009 (B) the population of the *Nosema*-infected bees started dropping after day 12 forward. Half of the population had disappeared after 19 and 50 days for the infected and non-infected group, respectively. Presumed onset of the disease in both years was around day 15.

VI. 4.2. Registration of activity

In order to find out if there were different activity levels between the two treatment groups, we investigated the number of flights that every bee undertook during the different weeks of its life (**Fig. 8**). In 2008 the activity of the Nosema+ bees was found to be significantly higher compared to the Nosema- bees during the fourth week (day 22-28, $p \leq 0.007$, Mann-Whitney-U-Test) which equals the second week after the presumed onset of disease (day 15). We also observed an increase in activity during week three in 2009, the week of expected disease onset. However, this difference however was not statistically verifiable ($p = 0.22$). During this period of time (day 22-28 in 2008 and day 15-21 in 2009) a decrease of the experimental populations of the N+ bees was observed which was especially pronounced in 2009. A trend towards higher activity of the N+ group was also found during the subsequent weeks in both experiments, but these differences were not statistically verified in either year.

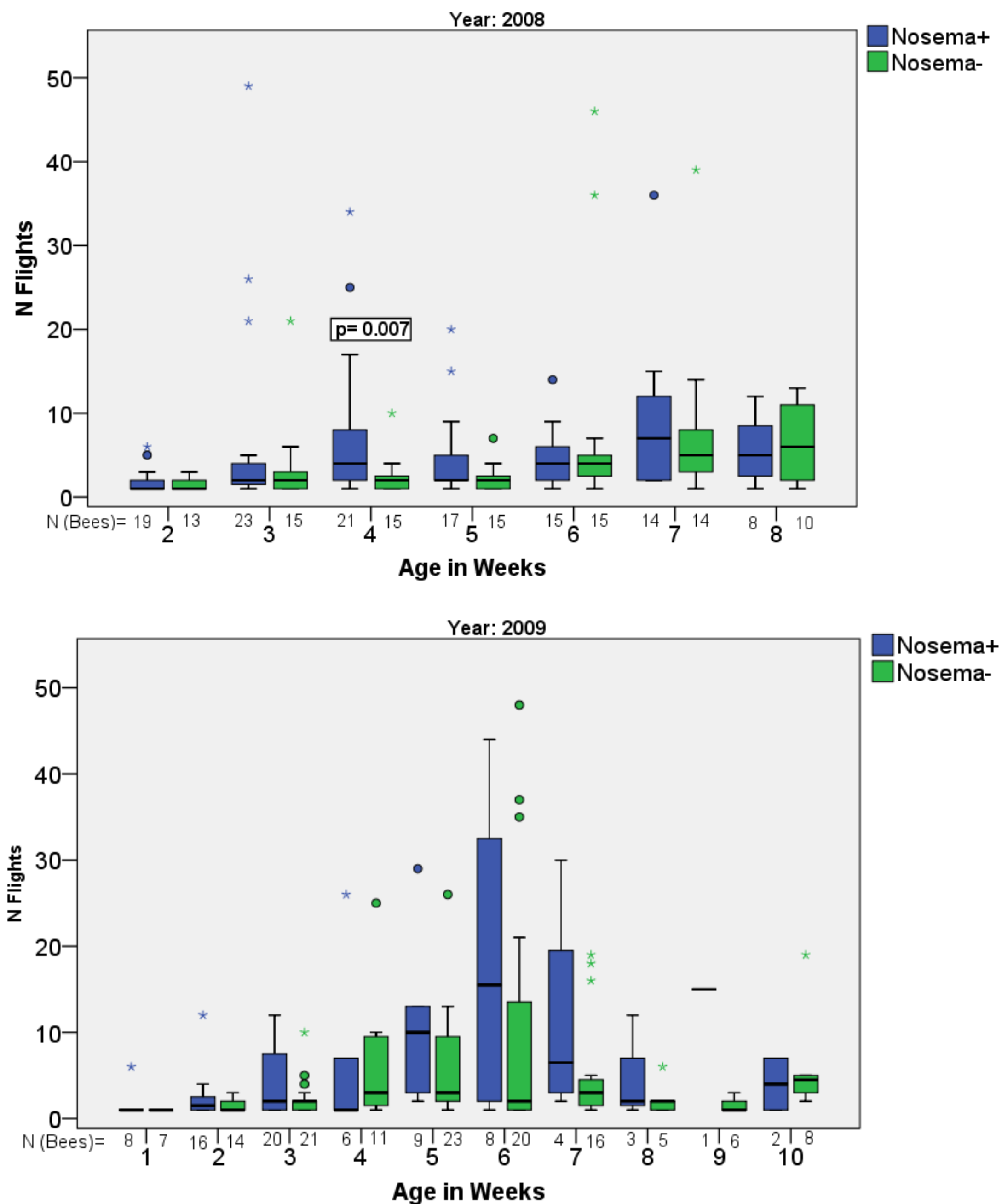

Figure 8: Comparing the activity of infected and non-infected workers at the hive entrance per week of life. Plotted is the activity per bee during the experiment in 2008 (above) and the experiment in 2009 (below). A significantly increased activity for the inoculated bees was observed during week 4 in 2008. In 2009, a non-significant trend towards increased activity was detected for the inoculated group during week 3. In the following weeks in both years there is a tendency towards more activity in the infected group but this difference was not statistically verifiable. Number of bees (N bees) contributing to each group is shown below the respective box. (o= outlier; asterisk= extreme outlier)

VI. 4.3. Time spent outside the hive

During 2008a total number of 1230 (Nosema+: 696, Nosema-: 534) flights was observed, in the following year this number was 1400 (Nosema+: 545, Nosema-: 755). When comparing the time duration the infected and non-infected bees spent outsid the hive during the different weeks of their life (**Fig. 9**), a significantly longer time of absence was found for the infected bees during weeks three ($p \leq 0.007$, Mann-Whitney-U-Test), four ($p \leq 0.021$), and five ($p \leq 0.04$) in 2008. Their time of absence was found between 1.6, 1.74, and 1.54 times longer compared to the non-infected bees. The median times the non-infected bees spent outside the hive were found to be relatively short for bees that would be expected to be foraging by then, ranging between 1.5 and 2 minutes. A similar effect on the infected and non-infected bees was not found during the experiment conducted in the following year. The time of absence for both groups was rather leveled during the first 5 weeks. During the 6th and 8th week of life longer foraging trip durations were recorded for the inoculated bees. Here, it was observed that the median time that an infected bee spent outside the hive was prolonged by a factor of 3.3 and 2.3, respectively, compared to non-infected bees. However, this difference was not found to be significant (6th: $p = 0.334$; 8th: $p = 0.2$), probably due to the low number of infected bees remaining in the colony (**Fig. 8, below**).

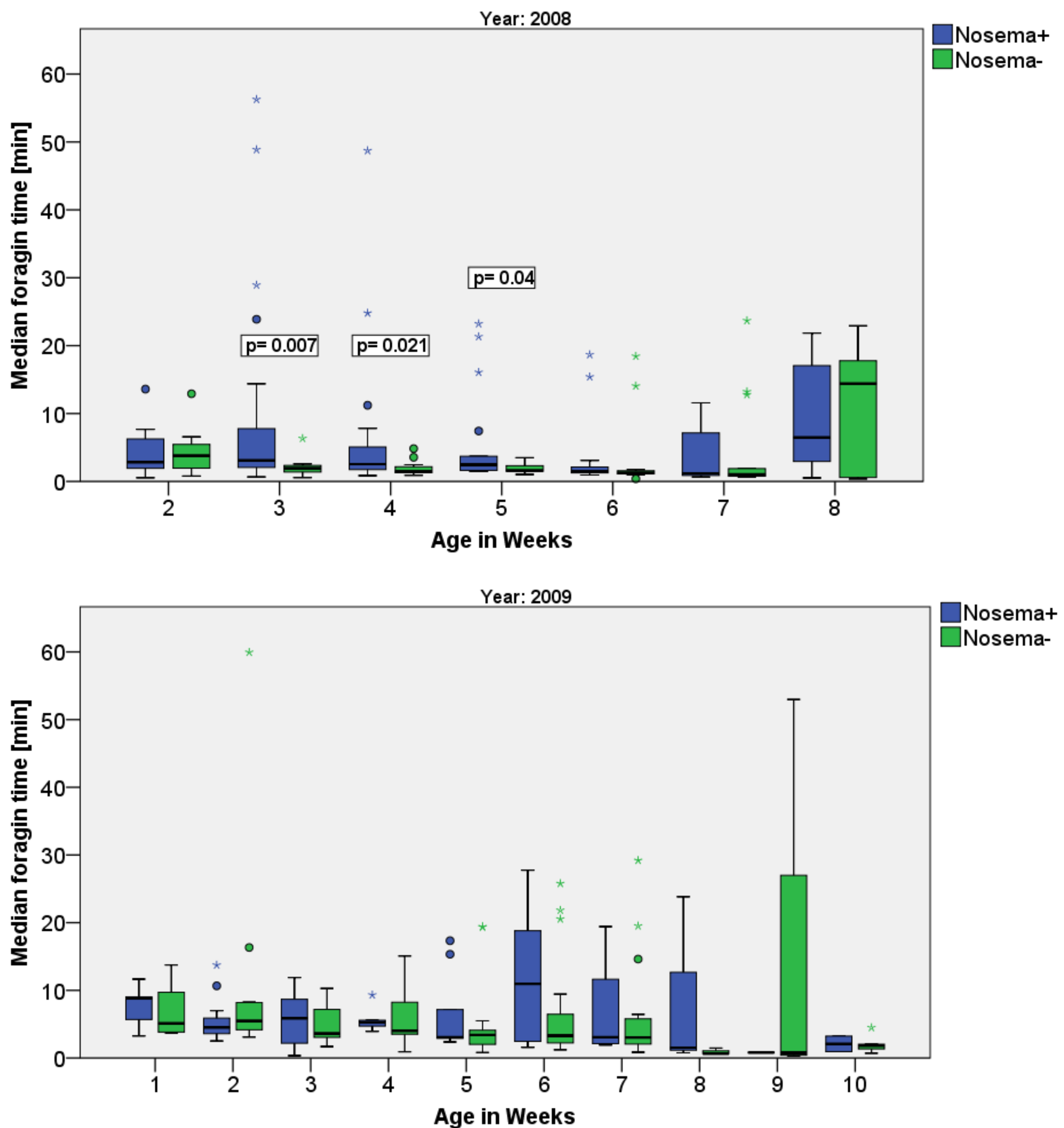

Figure 9: Comparing the period of time infected and non-infected worker bees spent outside the hive per week of life. Plotted is the median duration which infected and non-infected bees spent outside the hive (in minutes) over the experimental period in 2008 (above) and 2009 (below). During three consecutive weeks (week 3-5) in 2008 the inoculated bees spent significantly more time outside the hive compared to the non-inoculated bees, an effect that could not be observed in 2009. (o= outlier; asterisk= extreme outlier)

VI. 4.4. Dependence of increasing foraging activity on longevity

In order to test if the differences in lifespan found for the *Nosema* inoculated groups in both years were influenced by higher activity levels especially in week four (2008) and week three (2009) (see also **Figs. 8 and 9**), it was investigated if there was any significant dependence between both parameters. Thus, the total activity of a bee during these respective weeks (independent variable) was plotted against its final age (dependent variable) (**Fig. 10**). High activity during the investigated weeks (2008: Regression coefficient (**Rc**): -0.282, $p = 0.088$; 2009: Rc: -2.644, $p \leq 0.008$) were found between activity and final age for the inoculated groups, indicating that infected bees that were more active during these investigated weeks had a reduced lifespan. The reason that no significant dependence was found in 2008 is mainly due to one outlier (**Fig. 10**, above). Removal of this outlier would yield a significant regression for the inoculated group in 2008 (Rc: -0.718, $p \leq 0.001$). In contrast, no significant dependence was found for the control group in 2008 (Rc: 0.377, $p = 0.589$) or 2009 (Rc: 3.609, $p = 0.089$). T-test comparisons of both treatment groups in the respective years revealed a significant difference in 2008 ($T = 3.551$, $p \leq 0.001$) and in 2009 ($T = 2.51$, $p \leq 0.016$).

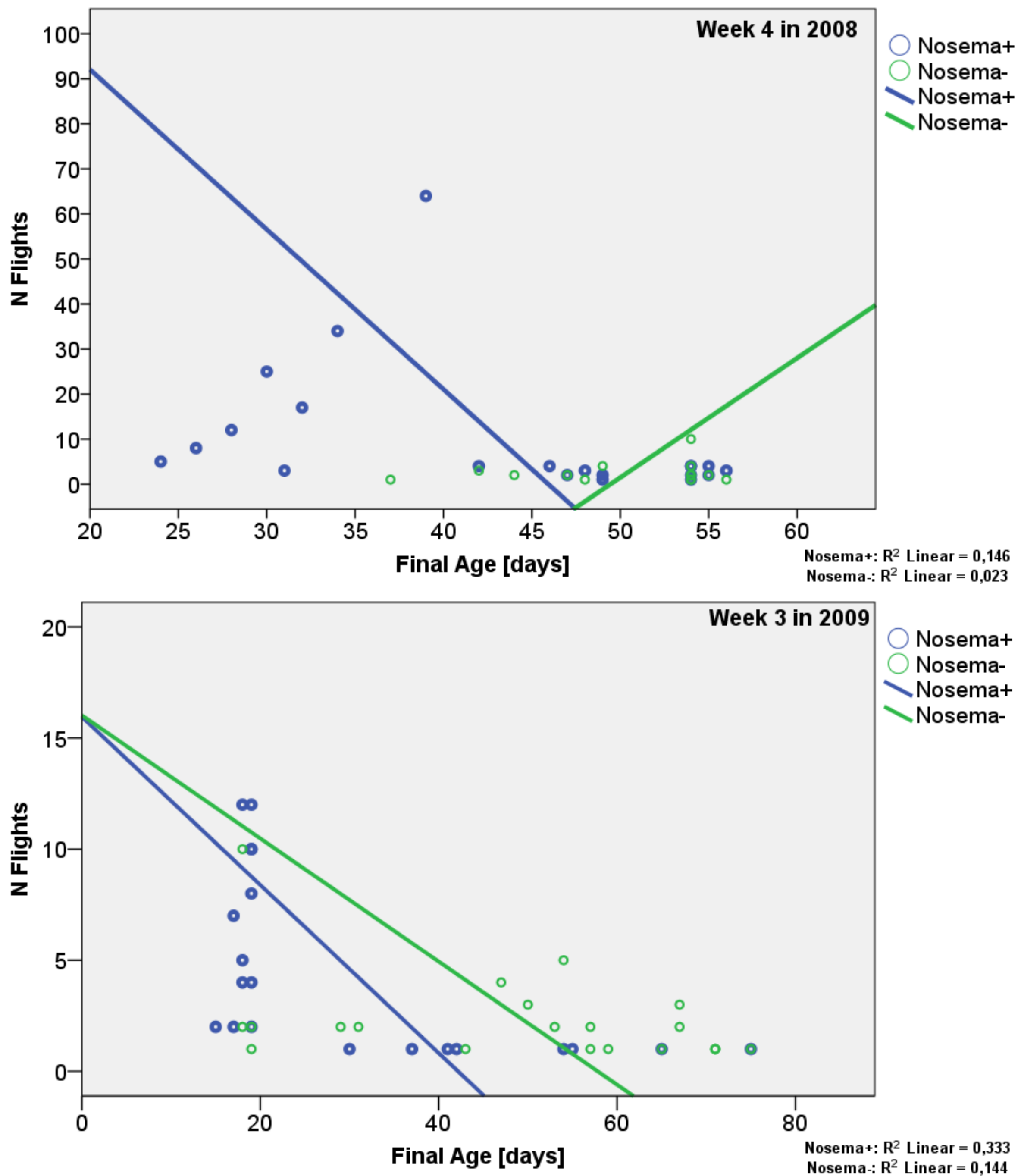

Figure 10 Relationship between activity and longevity in 2008 (above) and in 2009 (below).
Abszissa: final age at last registration. Ordinate: Number of flights in week 4 (2008) or week 3 (2009). Each point represents a single bee, solid lines give the linear regression of final age on activity (number of flights).

VI. 5. Discussion

The results presented in this study are in agreement with several studies conducted in the past on the basis of standard observations rather than modern monitoring technologies. The most obvious difference between infected and non-infected bees was the reduced duration of registration which was determined as lifespan. The finding that the lifespan is reduced for infected bees (-14%, 2008; -62% 2009) is in accordance with the results reported in studies by Maurizio (18% and 36.5%, Maurizio 1946), and Beutler and colleagues (24.3%, Beutler et al. 1949) and does also corroborate the observations of Borchert (Borchert 1928) who, after three weeks, found 24% and 21% of infected bees remaining inside of his test colonies compared to 50.9% and 73.4% of the control populations. In addition, in 2009 a drop in the experimental population of the inoculated bees could be observed around the time of the predicted disease onset (day 15) when the parasite is thought to have spread throughout the intestinal tract (Gross and Ruttner 1970).

It can be argued, that bees that were not registered at the hive entrance anymore might have drifted to other colonies in the perimeter. This can be assumed as rather improbable, since all colonies in a close perimeter of ten meters from the test-colony were all monitored by scanners, thus registering any activity of tagged drifters. The first non-monitored colonies were about 400 meters away making it rather improbable that bees would drift towards those, though not impossible. Furthermore, it could be argued that bees lost their transponders rather than got lost. Of course, there was always the possibility that a tagged bee did remove the tag but one would think that this would be more evenly distributed along both treatment groups. Since the median duration of registration was reduced for the Nosema+ group in both experimental years, though not significantly in 2008, the slope in the decrease of the infected bees was rather steep, especially in 2009, which make this assumption also rather improbable.

The differences between both experimental years concerning the lifespan of the infected bees might be due to the different success in the inoculation rate. In the experiment in 2008, the probability that a bee was successfully inoculated with *Nosema* was found to be around 76% with the majority (13 of 18 bees) showing only low degrees of infection according to Ritter (1996). In contrast, in 2009 all thirteen bees belonging to the additional positive control caught from the hive were infected with the majority showing a medium or high degree of infection (10 of 13 bees).

Of course, there is also the possibility that colony differences contributed to the annual variations in the results since the brood combs were producing the bees for these experiments originated from different colonies.

It was observed that *Nosema* inoculated bees spent more time outside the hive in 2008. This corroborates with findings by Kralj and Fuchs (Kralj and Fuchs 2009) that bees infected with *Nosema* sp. need more time to return after release from the close vicinity of the colony. Automatic registration passages at the hive entrance revealed higher flight activity especially during the fourth week of their life. Increased activity recorded early in life could add even more to losses due to inexperience of young bees in navigation. This was partly shown by investigating the relation between the increased activity in the weeks after onset of the disease and the lifespan of inoculated and non inoculated bees. Inoculated bees showed precocious foraging activity which resulted in an earlier disappearance from the colony, while the non inoculated bees were still rather inactive, resulting in a higher life expectancy. Wang and Moeller already reported that infected bees switch from nursing to other tasks more rapidly (Wang & Moeller 1970), probably due to less well developed hypopharyngeal glands and Hassanein (Hassanein 1953) found that infected bees start foraging earlier, with the time of the in-hive duties being reduced form 17-18 days (non infected) to 8-11 days. Taking higher risk by the increased early foraging of infected or injured workers from social insect societies is seen as compensation for the significantly shorter lifespan (M. Woyciechowski and Kozlowski 1998; Tofilski 2009; Moron, Witek, and Michal Woyciechowski 2008). Risk prone foraging could explain early losses of workers recorded in the infected group although impaired orientation (Kralj and Fuchs 2009) and learning (Kralj et al. 2007) certainly played a role.

In addition, it was also observed that some, mainly infected, bees with a low activity disappeared very early especially in 2009. This also might be explained by the differences in the degree of infection. Bees with a severe course of disease, a scenario more probable in 2009, could have died inside or outside the hive due to their stronger degree of infection providing them with an overwhelming physiological challenge, namely degeneration of the midgut and its metabolic function, thus disrupting the degradation proteins contained in pollen (Beutler, Opfinger, and Wahl 1949; Ritter 1996; Malone and Gatehouse 1998), less developed hypopharyngeal glands (Wang & Moeller 1969; Liu 1990), and more energetic stress, due to a

Nosema induced decline of the sugar level in the hemolymph (Mayack and Naug 2010). Another possible explanation for the early departure could be that strongly infected bees departed in order to isolate themselves from the colony before they die as was demonstrated in the moribund ants (Heinze and Walter 2010).

The results presented support the suicidal hypothesis (Smith-Trail 1980). Increased turnover indicated by early loss of diseased bees reduces infection in the colony and consequently lowers the transmission rate of the pathogen. Pronounced losses thus have a positive effect on the colony level, but could have a negative effect on the population level if infected bees enter other colonies and spread the pathogen. Changed behavior promoting losses also occurred by the parasitic mite *Varroa destructor* (Kralj and Fuchs 2006) indicating a general response to pests and diseases. The new evidence of self removal induced after extensive narcosis with CO_2 and the cytostatic drug hydroxyurea (Rueppell, Hayworth, and Ross 2010) and a similar response in ants affected by disease and chemicals (Heinze and Walter 2010) suggest that changes in behavior are neither restricted to diseases nor to honeybees. Contrary, modified behavior might be a very general response also to other stress factors in the broad group of social insects acting as a mechanism to protect nest mates from transmission of harmful pathogens or agents. Social withdrawal by self-removal was described as an adaptive altruistic trait serving to maintain inclusive fitness in social animals (Heinze and Walter 2010).

VII. Behavioral changes in adult honeybees being incubated at different temperatures during pupal development stage.

VII. 1. Summary

Maintaining a constant temperature inside the broodnest is an essential requirement for honeybee development especially during pupal stage. A temperature of approximately 34-35°C is thought to promote an optimal development. The usual temperature range found in the broodnest lies between 32°C and 36°C. Inside these thresholds, bees seem to develop normally in terms of morphological properties. Nevertheless, there have been only few studies on behavior and development of bees reared at sub- or above-optimal temperatures. Thus, the RFID method was used to monitor groups of bees reared at different temperatures (32°, 35°, and 36° C) over their life and record data about activity, foraging trip duration, and longevity.

We could show a strong correlation between brood nest temperature, flight activity and life span. It was observed that bees reared at above-optimal 36°C were overly active and spent most time outside the hive compared to the other two tested groups of bees (32°C, 35°C) tested. Bees reared at an optimal 35°C lived longer than the 36°C- and the 32°C-bees.

VII. 2. Introduction

VII. 2.1. Regulation of temperature inside the brood nest

Independent of differences in environmental temperatures honeybees are able to maintain a constant temperature inside their nest. This phenomenon of thermoregulation has already been known since the time of Aristotle (384-322 B.C.) (Koeniger 1978). Honeybees can either produce endothermic heat by dislocating their wings and simultaneously contracting their flight muscles (Esch 1960; Esch 1964; Esch and Bastian 1968; Esch and Bastian 1970) or reduce the temperature in the nest through several cooling strategies like ventilation by wing fanning, water evaporation, and even partial evacuation (Chadwick 1931; Lindauer 1954b). Using this abilities, bees can constantly keep the temperature in the brood nest in a range of 32 and 36°C (Himmer 1932; Jay 1963; Seeley 1985; Himmer 1927) during times of brood rearing, with a temperature optimum at around 35°C (Himmer 1927; Simpson 1961). To maintain this constant temperature throughout the brood area many individuals of the colony cluster together combining their individual heating effort (Esch 1960). With the introduction of thermographical observation techniques, individual heating behaviors have been discovered by Bujok and colleagues (Bujok et al. 2002) and Kleinhenz and colleagues (Kleinhenz et al. 2003). Bees either press their heated up thoraces on the wax caps of sealed brood cells, transferring the warmth into the cell beneath, or crawl into empty cells which are surrounded by occupied broodcells and by heating up inside these cells the heater bees are providing warmth to the surrounding cells.

As long as the temperature range of the brood nest remains between 32 - 36°C newly hatched bees do not show any morphological differences (Himmer 1927; Soose 1954; Seeley 1985; Groh, Tautz, and Rössler 2004; Tautz et al. 2003), but it is known that extreme deviations from this range lead to increased brood mortality and morphological malformations like crippled wings and extremities in newly emerged bees (Himmer 1927; Weiss 1962; Koeniger 1978; Groh, Tautz, and Rössler 2004).

Bees reared at threshold temperatures of 32°C and 36°C show behavioral and physiological differences compared to those bees reared at a temperature optimum. Taking into consideration that during the metamorphosis from larva to pupa the nervous system undergoes a process of complete remodeling as documented in

other holometabolous insects like *Manduca sexta* and *Drosophila melanogaster* (Levine and Weeks 1990; Truman 1992) and that an alteration of temperature affects the development of antennal lobes in *Manduca* (Rössler, Tolbert, and Hildebrand 2000), bees reared at threshold temperatures of 32°C and 36°C might show behavioral and neuro-physiological differences compared to those bees reared at a temperature optimum. Tautz and colleagues observed that bees reared at lower temperatures showed abnormal dance performances, lower probability of dancing and a reduced number of dance circuits, compared to the bees reared at high temperatures (Tautz et al. 2003). Furthermore, they showed that bees reared at the high temperature threshold performed significantly better during one-trial olfactory conditioning experiments used to assess learning ability and memory consolidation. Becher and colleagues (Becher, Scharpenberg, and Moritz 2009) supported the findings by Tautz by showing that bees reared at 32°C danced less frequently than bees reared at 34.5 and 36°C. They also observed a significant negative correlation between brood rearing temperature and onset of foraging: Bees of the 36°C group had the earliest foraging flights.

Analysis of sensory input regions of brains from honeybees reared at different temperatures, revealed differences concerning the number of synaptic circuitries in areas for olfactory input in the mushroom bodies (Groh, Tautz, and Rössler 2004). Bees reared at the temperature optimum (34.5°C) were found to possess significantly more synaptic complexes (microglomeruli) in this area compared to bees raised at +/- 1°C from this optimum.

An experiment by Bock, who used a previous model of the RFID-system employed in this study, showed that longevity and activity of bees reared at different temperature levels varied significantly (Bock 2005). Bees from the low (32°C) temperature group had the shortest lifespan while bees reared at high temperatures (36°C) lived the longest, some even surviving the winter period. The highest activity was detected for bees reared at optimal temperatures of 35°C followed by the 32°C-bees and the 36°C-bees. Since Bock used different bee-tunnel designs the detection rate of the transponders was negatively affected, the effective determination of the foraging duration of the tested bees was not possible.

Combining the RFID-system with an improved tunnel design, allowing a more effective registration, a similar experiment was conducted in order to acquire additional information about the time period bees spend outside of the hive and how

it alters over the lifetime of bees that were reared at different brood temperatures during pupal development stage.

VII. 2.2. Development and tasks of the Honeybee *Apis mellifera*

Honeybees belong to the holometabolous insects meaning that they undergo a complete metamorphosis over four different development stages: egg, larva, pupa, and finally imago, the adult animal. The development of a worker bee commences by deposition of a fertilized egg into an empty brood cell by the queen bee. In the following this development is described representative for the European honeybee.
During the three days after deposition an embryo develops inside of the egg. Subsequently, the larva hatches from the egg and is provided with food, a mixture of honey, pollen, and hypopharyngeal gland secrete, by nursing bees. During the following six days of the larval development stage the larva grows, gains weight, and undergoes four molts while the broodcell is still uncapped (Jay 1963). About eight to nine days after egg deposition the feeding of the larva ceases and the brood cell is sealed by a wax lid. This marks the beginning of the pupal development stage. After the sealing the larva spins itself into a silken cocoon which elicits the transformation into the pupa. During the following 10-11 days the complex transformation from pupa to imago is completed. In this process, a set of cells, previously inactive, is activated and quickly begins with cell division, forming the different tissues of the imago. Twenty-one days after egg deposition (Jay 1963) the young imago opens the wax lid of its nursery with its mouthparts and emerges into the colony.
The tasks executed by honeybees are more or less determined by age. In cohort experiments, a cohort being a group of equally aged bees, Seeley (Seeley 1982) observed different activities by different age groups of bees. During the first days of their adult life, young bees tend to clean cells, eat pollen, and rest (latter can fill about 20% of the time). About three to four days after hatching the gradual development of their hypopharyngeal glands allows them to perform nursing duties for the brood. Other hive tasks include provision of the queen, capping of broodcells, and the cleaning and feeding of nestmates. At an age of around twelve days bees take on tasks in the periphery of the hive which include nectar and pollen processing, guard duties, and foraging, with the bulk of the foraging activity being observed after about three weeks and continuing until death. It has to be kept in mind that this age

polyethism is not a rigid framework. It was observed in well-supplied colonies. The needs of the colony affect the tasks of workers.

VII. 3. Material and Methods

VII. 3.1.Experimental Procedure

On July 21, 2009, the preparations for the broodtemperature experiment started with the caging of queens from three different colonies (location: Würzburg University) on three empty brood combs. Subsequently, the caged combs were hung back into their respective colony for 24 hours. During this time period, the queens were still attended by nursing bees which fitted through the gaps of the cage bars, but the egg deposition of the queens, unable to leave their cages, was limited to the brood combs on which they were fixated. Thus, the age of the hatching bees from these combs did not differ by more than a day. The cage was removed on July 22, 2009, allowing proper brood care inside the colony during the next ten days until cell capping. On August 1, 2009, the cells were mostly capped, leading to the extraction of the combs from the hives. To guarantee some genetic variance each brood comb was divided into three parts. One third of every comb was assigned to a different rearing temperature. The 3 x 3 comb pieces were embedded into an empty honeycomb, one for each temperature group, to provide the freshly hatched bees with food (**Fig. 11, left**). Afterwards, the combs were placed into incubators of identical construction (model: Memmert IPP 500, Memmert GmbH + Co. KG, Schwabach, Germany, **Fig. 11, right**). The temperature and humidity of each incubator was checked by a USB thermal sensor (EL-USB-2-LCD, Lascar Electronics Inc.) every five minutes. Immediately after hatching the young bees were tagged with RFID-transponders. A total number of 600 bees was tagged, two-hundred per temperature group. The tagged bees were introduced into a registration hive (Mini-Plus, Bienenzuchtbedarf Heinrich Holtermann GmbH & Co KG, Brockel, Germany), containing bees from the *Apis mellifera carnica* breeder line. In order to avoid overly hostile reactions of the hive bees towards the tagged newcomers, the RFID-labeled bees had to be introduced carefully into the registration hive. We adapted a similar approach that apiarists use in order to introduce new queens into their colonies. A number of 100 bees were housed in two stainless steel cages after the transponders were attached.

The dimensions of the cages were 69 x 85 x 44mm (height x width x depth). Over a circular opening on the ceiling (diameter: ≈17mm) the bees were inserted into the cage. A sliding acrylic glass window allowed observations of the bees inside the cage as well as a quick removal e.g. if they got rid of their transponder.

Figure 11: **left: Pieces of brood combs from different hives embedded into a honey comb and framed in a cage to keep the freshly hatched bees confined on the comb. right: Memmert IPP 500 incubator used to store the combs.**

The floor plates of the cages were dotted with small openings (diameter: ≈2mm) for air circulation. After all bees had been inserted, the openings on top of the cages were sealed with a layer of sugar dough a mixture of sugar and honey with a ratio of 10 : 3.5, w/w). Thereafter, the cages were hung upside down into the registration colony, framed by two stacked Brother Adam feeders, so that the sugar dough covered opening was positioned closely to the top edge of the combs, easily accessible for the hive bees (**Fig. 12**). The idea of this confinement was that the bees inside the cage would adopt the colony odor until the sugar dough was consumed, thereby minimizing possible hostile reactions when they finally departed from the cage into the colony. Data collecting began from the moment the first bees had been introduced into the colony.

Figure 12: Two stainless steel cages plugged upside down into a Brother Adam Feeder on top of the registration hive.

VII. 3.2.Data analysis:

Like in the experiments before calculations and analyses of the scanner data were done with SPSS 17. The transponders for the different temperature groups were read in and saved the day before the tagging began. Four main parameters were analyzed: I) The lifespan of every tagged bee which was defined as the period from hatching until the day of the last registration. II) Decline of the experimental populations over time. III) The activity at the hive entrance, where each movement either directed out- or inward was counted as one activity. IV) The time period spent outside of the hive. Here, a filter was used to search only for times that were longer than 50 seconds which is within limits of the shortest orientation flights (48s) measured by Capaldi and colleagues (Capaldi et al. 2000). This helped to erase many short-duration stays outside the hive which possibly occur during guard duties and quick in and out movements

The lifespan in days for the three temperature groups was determined and then tested for normal distribution with Shapiro-Wilk-Test. If the data were normally distributed, the mean lifespan of the different groups was compared by oneway ANOVA. If the result was found to be significant, a pair wise analysis was conducted using the Bonferroni-post-hoc-test. If the data was not found to be normally distributed a non-parametrical Kruskal-Wallis-test was conducted. Here a significant result led to a pairwise analysis using the Mann-Whitney-U-test. Activity per bee and the median time spent outside the hive were compared in a similar fashion. In addition to the average lifespan, the decrease of the experimental populations over

time was observed by Kaplan-Meier survival analysis. The pairwise comparison of the survival curves was done with Breslow –test (SPSS Statistics 17).

VII. 4. Results

Analysis of the temperature data, received from the USB-thermal sensors inside the incubators revealed that the temperature conditions did not vary from the set temperatures during the time the combs spent inside (**Fig. 13**). The mean temperature and humidity for Incubator #2, containing the 35°C comb until the date of hatching (August 11/12, 2009), was 34.98°C +/- 0.12[1] and 70.18% +/-5.06, respectively. Incubator #3 containing the 32°C comb until August 13/14, 2009 held a mean temperature and humidity of 31.93°C +/-0.22 and 77.1% +/-4.3, respectively, and incubator #4 containing 36°C comb until August 12/13 2009 held a mean temperature and humidity of 35.98°C +/-0.22 and 77.24% +/-5.6, respectively.

Since the different groups hatched on different days it was not possible to directly compare them for morphological differences. Nevertheless, no obvious morphological differences, e.g. deformed wings were observed immediately after hatching of the young imagines.

The first bee being registered on the afternoon of August 14, 2009 belonged to the 36°C-temperature group. On the evening of the same day the first bee of the 32°C-temperature group was registered. On the afternoon of the following day (August 15, 2009) the first bee of the 35°C-temperature group was registered. The last registration of a labeled bee took place after 84 days on the November 11, 2009. During this period of time 570 bees (95%) of the 600 originally introduced individuals were registered at least once at the entrance of the hive. These bees split into the three temperature groups as follows:

32°C: 194/200 (97%)

35°C: 183/200 (91.5%)

36°C: 193/200 (96.5%)

[1] Standard Deviation of the mean

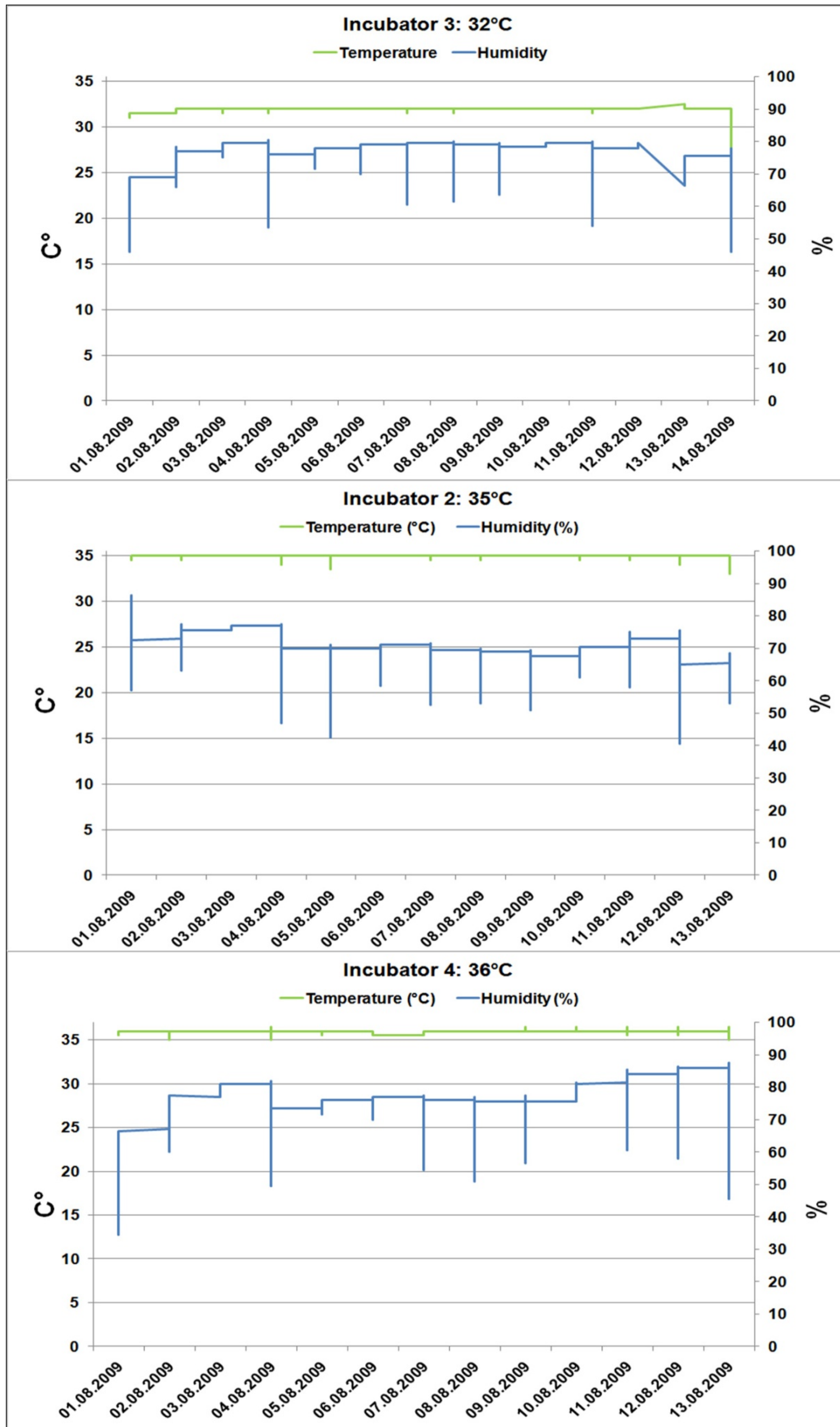

Figure 13: **Overview of the temperature- and humidity-monitoring inside of each incubator.** Short-lived drops of temperature and humidity are due to opening of the incubator door in order to check temperature and humidity conditions on the LCD display of the USB thermal sensor (EL-USB-2-LCD, Lascar Electronics Inc.).

VII. 4.1. Monitoring of Lifespan

Bees raised by 35°C during their pupae phase were registered the longest, followed by the 36°C-group and the 32°C group. Analysis of the lifespan inside every group by Shapiro-Wilk-Test showed that only the results for the 36°C-group were normally distributed ($p = 0.065$), while the lifespans of the other groups were not ($p < 0.001$). Therefore, the statistical tests used were the non-parametrical Kruskal-Wallis- and Mann-Whitney-U-tests. The median time the 35°C, 36°C-, and 32°C-bees stayed in the colony before their disappearance was 23, 22, and 21 days, respectively (**Fig. 14**). Though obviously not that large, the age difference between the groups was found to be significant ($p \leq 0.048$, Kruskal-Wallis-test). The pairwise analysis revealed a significant difference between the 35°C- and the 32°C-groups ($p \leq 0.029$), as well as between the 35°C- and the 36°C-groups ($p \leq 0.049$). No difference was found when comparing the lifespan of the 32°C- and 36°C-groups ($p = 0.432$).

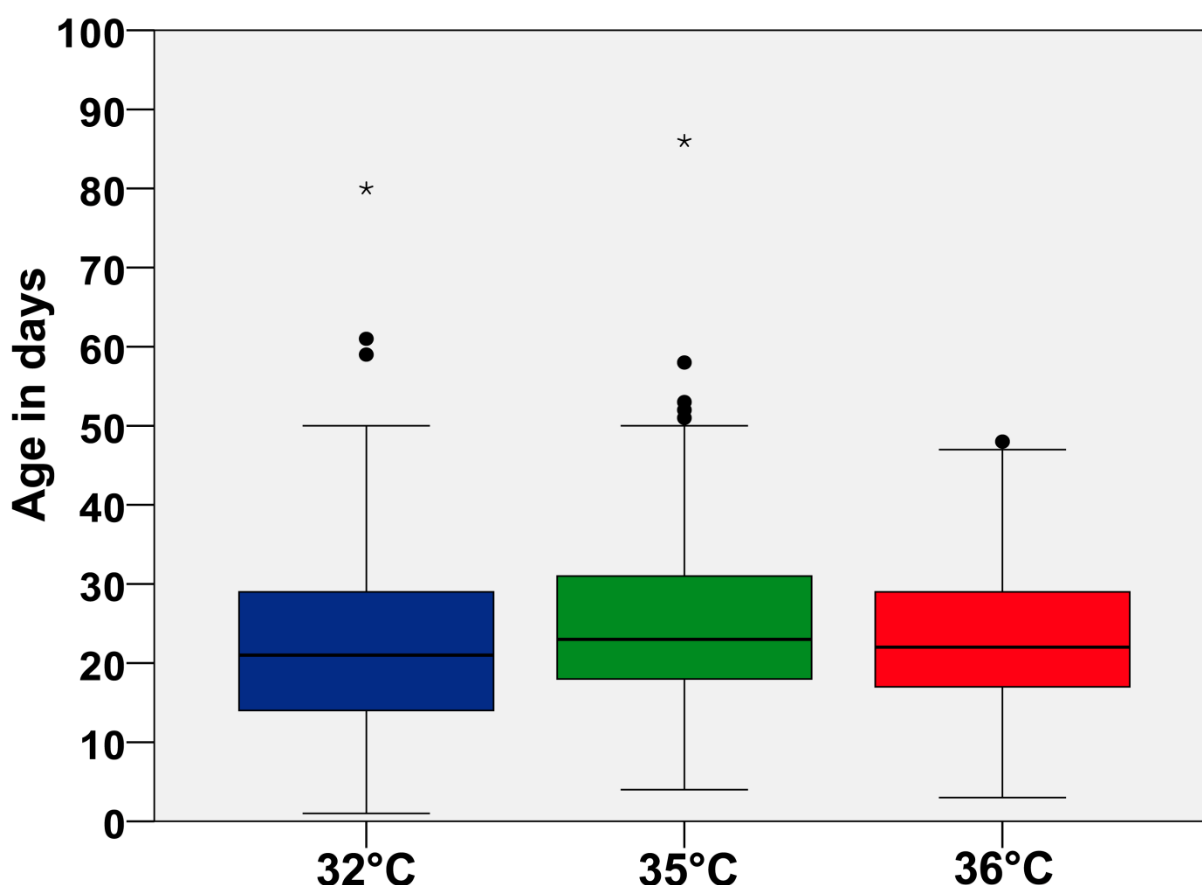

Figure 14: Distribution of the lifespan (in days) of the bees reared at the three temperatures. Plotted is the time until the last registration for every bee in the experiment. Bees reared at an optimal 35°C during pupal development have the longest lifespan, followed by the bees reared at an above-optimal 36°C and sub-optimal 32°C. (● = outlier, single asterisk = extreme outlier)

In addition, it was determined whether the bees that were not registered anymore disappeared outside of the colony or rather died at home. Therefore, the last set of registrations was examined to determine the direction the bee was heading to, when ultimately registered. It was found that the majority of bees (73.16%, N= 417) left the colony on their last registration. Less than ¼ had their last registration while entering the hive (22.28%, N= 127). For 4.56% (N= 26) it was not discernible whether they had entered or left the colony according to a missed registration at one of the successive antennas of the scanner. The proportion for the different temperature groups are shown in **Table 3**.

Table 3: Determination of the direction by bees from the different temperature groups after their last registration.

T-group	directed outward		directed inward		not definable	
	%	N	%	N	%	N
32°C	74.74	145	18.04	35	7.22	14
35°C	71.58	131	25.68	47	2.73	5
36°C	73.06	141	23.32	45	3.63	7

Interestingly, no bee of the 36°C-group was registered longer than 48 days. From the bees raised by 32°C five individuals were registered 50 days and longer, with one bee reaching the maximum of 80 days. Eight individuals from 35°C-group were registered 50 days and longer, with one bee reaching the maximum of 86 days.

Observation of the experimental populations showed a decrease of the population by 26.29% for the 32°C-group, 19.69% for the 36°C-group, and 12.57% for the 35°C-group during the first two weeks (**Fig. 15**). The majority of the missing bees at that time had their last registration on the way out of the colony (32°C: 78.43%, 36°C: 71.05%, 35°C: 65.22%).

A comparison by the Breslow-test revealed that the survival curves of the 35°C- and the 32°C-groups, as well as the 35°C-and the 36°C-groups are significantly different from each other (**Tab. 4**). In both cases, the 35°C-curve lies above the other two for most of the time. No difference was found when comparing the 32°C- and the 36°C-groups (p= 0.432).

Table 4: Results of the pairwise comparison of the survival curves using the Breslow Test.

		Chi^2	p-value
35°C	32°C	4.775	0.029
	36°C	3.888	0.049

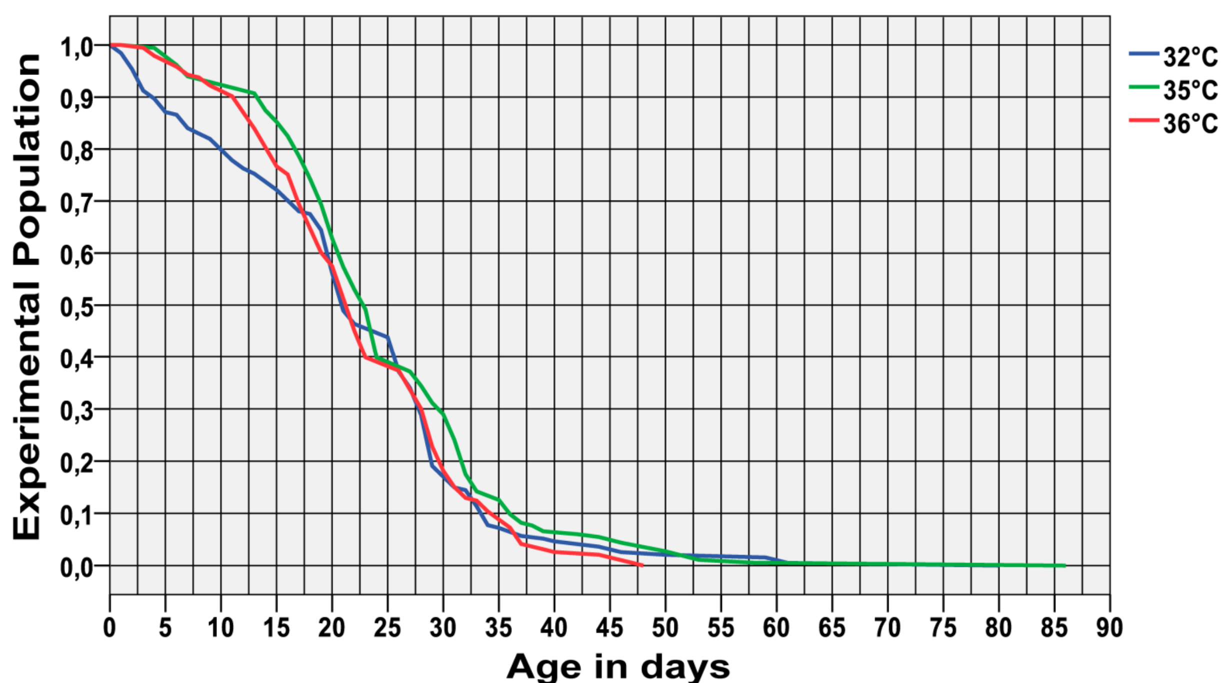

Figure 15: Decrease of the three experimental populations over time. An obvious decrease compared to the other groups was observed during the first two weeks of life of the 32°C-population. Half of the population had disappeared after 21, 22, and 23 days for the 32°C-, 36°C-, and 35°C-group, respectively.

VII. 4.2. Registration of Activity

At the end of the first week of life, the total activity in all temperature groups was relatively low. The 35°C-group showed the least activity during this time, with only 16 bees (8.7% of total N of bees registered) being registered at the hive entrance compared to 51 (32°C, 26.3%) and 67 (36°C, 34.7%) in the other temperature groups (**Tab. 5**). The bees reared at 36°C showed the highest activity of all groups up to the fourth week, while the 32°C-group already reached its highest level of total activity at the end of the 2nd week (**Fig. 16**). The total activity of the bees reared at 35°C increased steadily until it reached its maximum at the 5th week.

Table 5: Number of active bees (N) during the first six weeks of life.

	Week					
	1	2	3	4	5	6
	N active bees	N active bees	N active bees	N active bees	N active bees	N active bees
32°C	51	162	146	122	72	22
35°C	16	179	159	123	73	26
36°C	67	186	167	113	72	24

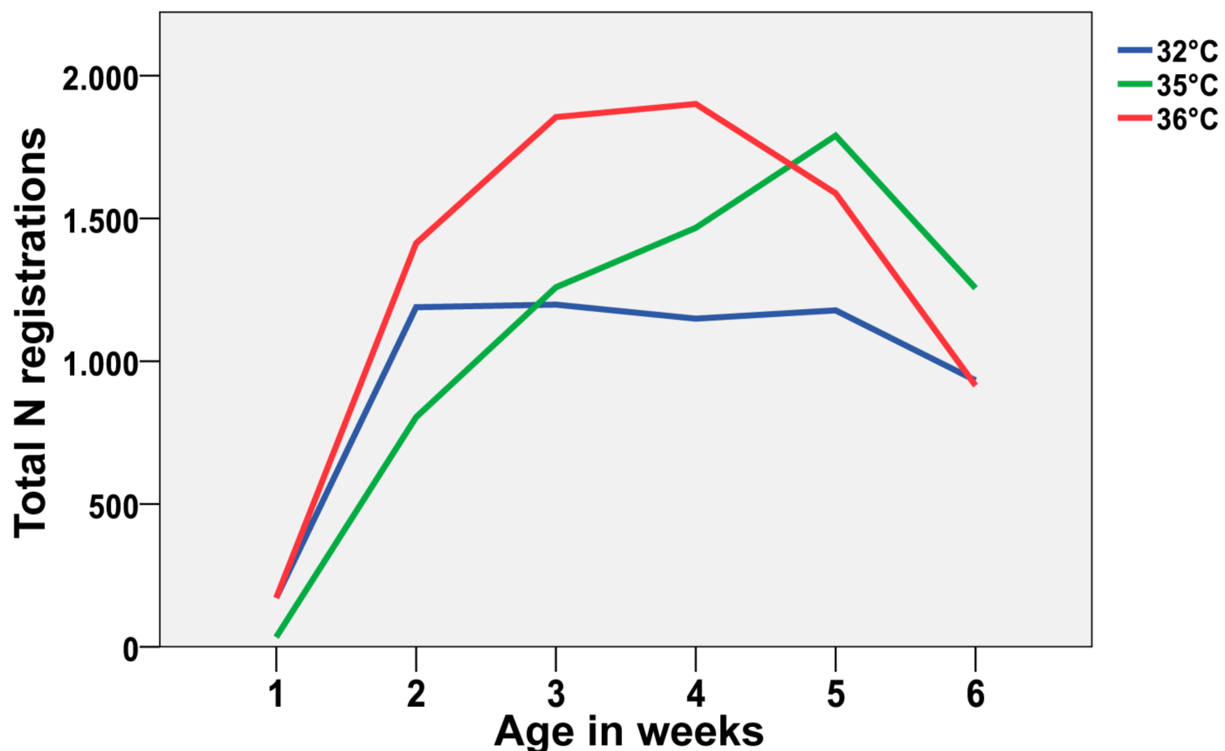

Figure 16: Total activity of the three temperature groups over the first six weeks of life. During the second week activity started increasing in all three groups, with the highest total activity registered for the 36°C-bees and the lowest total activity for the 35°C-bees. After the second week the activity level of the 32°C-remains constant while the activity level of both other groups rises steadily until week 4 (36°C) and 5 (35°C) before dropping again.

When looking at the activity per bee a similar picture as for the total activity per temperature group was found (**Fig. 17**). After the first week, no statistically significant difference was found between the three temperature groups. During the 2nd week, a significant difference in activity was found for the first time ($p \leq 0.002$, Kruskal-Wallis-test). Bees belonging to both the low and the high temperature groups showed a significantly higher activity compared to the bees belonging to the 35°C group ($p_{32°C} \leq 0.002$; $p_{36°C} \leq 0.002$, Mann-Whitney-U-Test). During the 3rd and 4th week, the activity of the 32°C- and 35°C-groups were on the same level (3rd: = 0.260; 4th: $p = 0.142$, Mann-Whitney-U-Test), while the 36°C-bees showed significantly more activity than both other groups (Kruskal-Wallis-test: 3rd: $p \leq 0.012$; 4th: $p \leq 0.001$; Mann-Whitney-U-test: 3rd: $p_{32°C} \leq 0.004$, $p_{35°C} < 0.05$; 4th: $p_{32°C} \leq 0.001$, $p_{35°C} \leq 0.01$). In the following 5th week, both the bees reared at 35°C and 36°C had 80% more activities registered (referring to the median) at the hive entrance compared to the bees reared by 32°C (Kruskal-Wallis-test: $p \leq 0.001$; Mann-Whitney-U-test: $p_{35°C} \leq 0.001$, $p_{36°C} \leq 0.003$), but did not differ from each other significantly. After the 6th week, no significant differences between the groups were found ($p = 0.766$, Kruskal-Wallis-test).

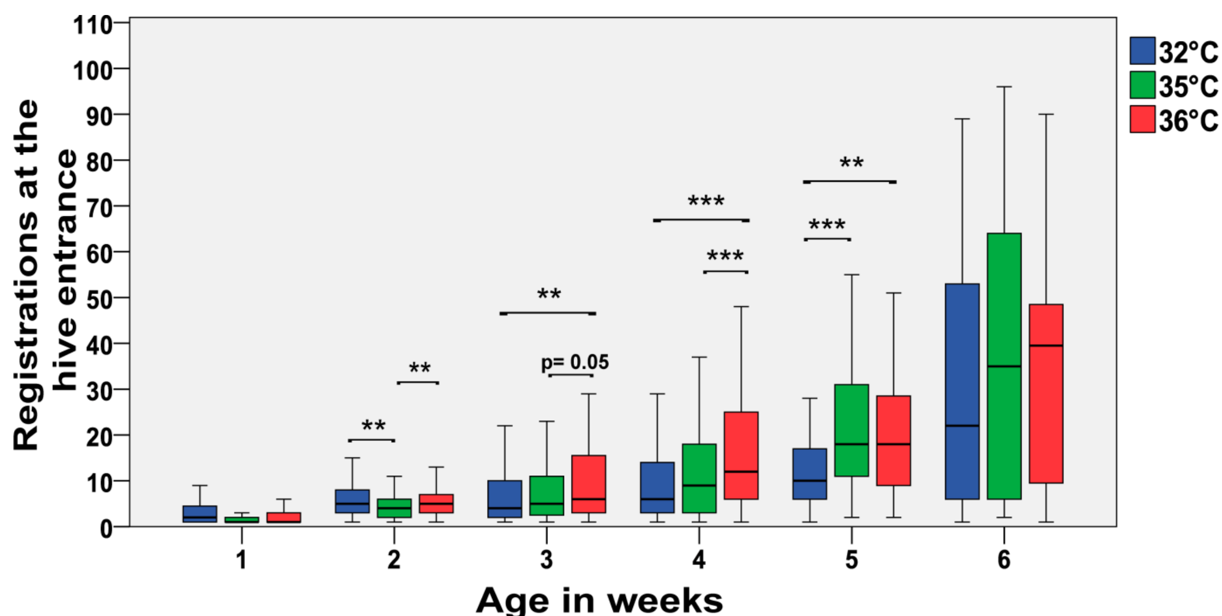

Figure 17: Comparing the activity of every bee from the different temperature groups at the hive entrance per week of life. Plotted is the activity (frequency of registrations at the hive entrance) per bee for every temperature group during their first six weeks of life. The same trend was observed as in the comparison of the total activity. The 32°C-and 36°C bees were significantly more active than the 35°C-bees during the second week. Over the next two week (3 and 4) the above-optimal reared 36°C bees were found to be the most active ones. During week 5 the 35°C-and 36°C-bees were both significantly more active than the 32°C-bees which seemed to be the less active group over the whole course experiment. *= p< 0.05, **= p< 0.01, ***= p<0.001

VII. 4.3. Time spent outside the hive

During the entire experimental period, 11290 stays outside the hive were calculable, including 10220 during the first six weeks (**Tab. 6**). In this period, most trips outside the hive were undertaken by the 36°C-bees (3992), followed by the 35°C- (3275), and the 32°C-bees (2953). In accordance with the results of the activity analysis, a low number of trips outside the hive were recorded during the 1st week of life. Most trips outside the hive by the low- and high-temperature group were made during the 4th week. The 35°C-group reached the peak for trips outside the hive during the 5th week. The decline of the experimental populations during weeks five and six corresponded with the decline of the number of trips.

During the first two weeks of life, it was observed that the majority of the flights rarely exceeded 10 minutes, with the medians for the 1st and 2nd being 6.86 and 4.38 minutes for the 32°C-group, 6.75 and 4.14 minutes for the 35°C-group , and 6.58 and 4.9 minutes for the 36°C-group (**Fig. 18, Tab. 7**). After the 3rd week, the duration of the trips started to get longer, which continued throughout the following experimental period. Analyses with the non-parametrical Kruskal-Wallis-Test showed significant

differences in the times spent outside of the hive from week two until week six ($p \leq 0.001$). Beginning with the 2nd and except for the 4th week, the bees reared at 36°C spent significantly more time outside the hive during their trips compared to the other two temperature groups (**Tab. 7 & 8**). Over the experimental period, the discrepancy of time spent outside the hive between the 32°C- and the 36°C-groups increased steadily. In comparison to the bees reared at 32°C, the 36°C-bees spent 11.9% more time outside the hive (related to the medians) in the 2nd week, 69.9% in the 3rd week, 74.1% in the 4th week, 72.2% in the 5th week, and 100.9% in the last week. Comparing the time of the 36°C- and the 35°C-groups showed that in all but one week (week four) the "hot" bees spent more time outside the hive: 2nd week: 18.4%, 3rd week: 40%, 4th week: -7.5%, 5th week: 16.2%, 6th week: 41.6%. During the third week a strong, though not significant ($p = 0.056$) tendency towards longer trips outside the hive was found for the 35°C-group compared to the 32°C-group. From week four to week six the 35°C-bees spent significantly more time outside of the hive than the 32°C-bees (**Tab. 8**): 4th week: 88.2%, 5th week: 48.3%, 6th week: 41.9%.

Table 6: **Number of calculable trips (N Trips) made outside the hive during the first six weeks of life.**

	Week						Total
	1	2	3	4	5	6	
	N Trips						
32°C	**86**	**636**	**493**	**697**	**484**	**557**	**2953**
35°C	**30**	**538**	**402**	**833**	**878**	**594**	**3275**
36°C	**139**	**823**	**666**	**1238**	**711**	**415**	**3992**
Total	**255**	**1997**	**1561**	**2768**	**2073**	**1566**	**10220**

Table 7: **Median duration in minutes spent outside the hive.**

	Week					
	1	2	3	4	5	6
	Median [min]					
32°C	**6.86**	**4.38**	**4.72**	**5.67**	**11.35**	**8.47**
35°C	**6.75**	**4.14**	**5.73**	**10.67**	**16.83**	**12.02**
36°C	**6.58**	**4.90**	**8.02**	**9.87**	**19.55**	**17.02**

Table 8: Results from pairwise comparison of the times the bees spent outside the hive (Mann-Whitney-U-Test). Significance level: 0.05

		Week					
		1	2	3	4	5	6
		p-value					
36°C	32°C	0.839	0.001	< 0.001	< 0.001	< 0.001	< 0.001
	35°C	0.307	< 0.001	0.003	0.017	0.001	< 0.001
35°C	32°C	0.436	0.151	0.056	< 0.001	< 0.001	< 0.001

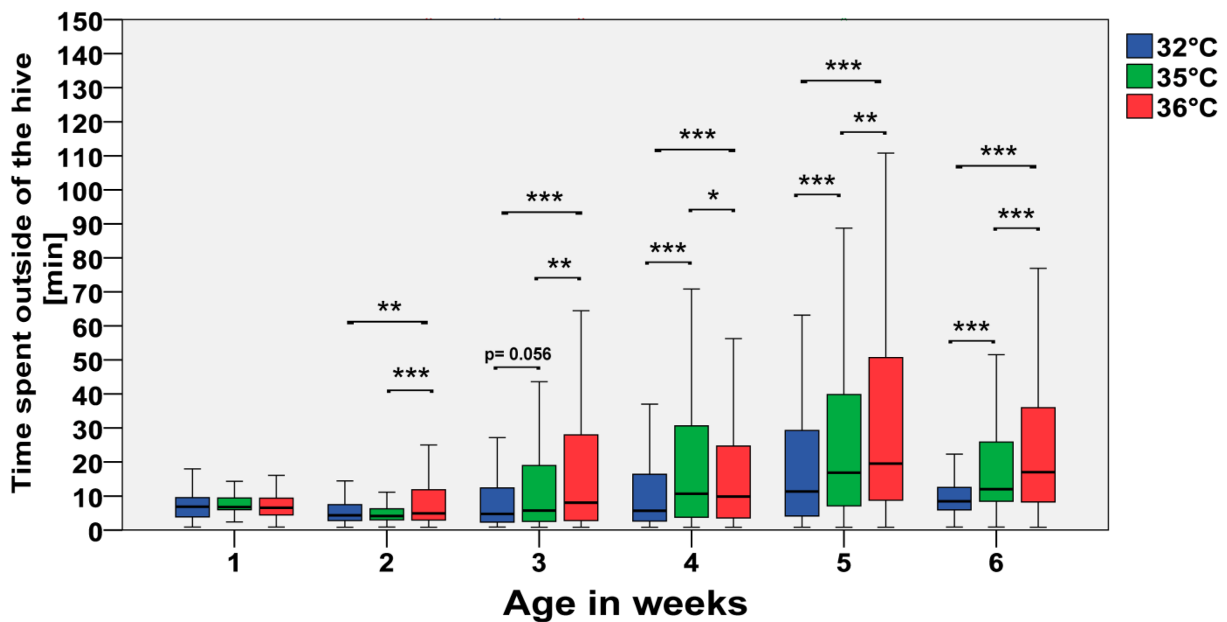

Figure 18: Comparing the period of time bees of the three temperature groups spent outside the hive per week of life. Plotted is the median duration which bees of the three temperature groups spent outside the hive (in minutes) over the first six weeks of their life. The bees reared at 36°C spent the longest periods time outside of the hive with exception of week 1and 5 while the bees reared at 32°C made the shortest trips throughout almost every week.

For better overall view on the boxes and whiskers the outliers were removed (see **Figure S5** in the Supporting Information). *= p< 0.05, **= p< 0.01, ***= p<0.001.

VII. 5. Discussion

Even though the temperature range of 32-36°C found in brood areas in times of brood rearing does not affect the morphology of the honeybee (Himmer 1927; Soose 1954; T. D. Seeley 1985; J. Tautz et al. 2003; Groh et al. 2004), the presented results add additional information on their effect on vitality and behavior (Tautz et al. 2003; Bock 2005; Becher, Scharpenberg, and Moritz 2009). The sub- and above-optimal brood rearing temperatures during the temperature sensitive pupal development stage (Koeniger 1978) differently affected the longevity, activity, and time spent outside the hive.

Bees reared at an optimal 35°C were found to be registered for the longest period of time, while bees reared respectively at sub-optimal 32°C or above-optimal 36°C were registered for a significantly shorter period of time, even though the median duration differed only by one and two days, respectively. In a similar experiment conducted by Bock (2005), it was also found that the bees reared at 32°C had the shortest mean registration duration compared to the other two groups. In Bock's study, though, the 36°C-bees were registered for the longest time period followed by the 35°C. This was also due to the fact that a quarter of the bees from the above-optimal group were the only ones being registered again after the winter period, a feat that could not be confirmed for any group during this study, even though the experiment did start at the exactly same time of the year. One could hypothesize about possible reasons for that. First of all, Bock used bees from three different colonies with each colony accounting for a single temperature group only, while the temperature groups in this experiment were a mixture of three different colonies. Genotypic differences between the colonies could have led to the development of winter bees rather than the different temperatures.

Another factor affecting the differences might have been different weather conditions. Since we did not find any tagged bees at the beginning of the following spring, it was not possible to test Bock's hypothesis that temperature could play a role in the production of long-lived winter bees. The requirements for the development into winter bees are thought to be correlated with several factors. The reduction of brood production was found to lead to hypertrophied hypopharyngeal glands and increasing fat bodies (Maurizio 1950; Maurizio 1954; Fluri & Bogdanov 1987 as reviewed in Amdam & Omholt 2002) when no extensive nursing duties are necessary. These physiological changes increase the protein status (amount of available protein) in

haemolymph, fatbody, and hypopharyngeal gland. One of these proteins, the very high-density-lipoprotein (VHDL) vitellogenin with its antioxidant function (as reviewed in Amdam & Omholt 2002) is thought to play a crucial role concerning the honeybee longevity. Furthermore, foraging is a risky task leading to a reduced lifespan (Visscher and Dukas 1997; Tofilski 2009). Considering this, it can be assumed that the moderate temperatures during late summer and fall of 2009 did not stop the hive from producing brood, not allowing the physiological changes to occur that are necessary for winter bees. Furthermore, the bees continued foraging in order to find supplies for the colony, leading to the high rate of bees from all tested temperature groups, that never returned from a foraging trip (73.16%) An unusually temperate autumn in 2009 led to extended activity until early/mid October. Instead of staying inside the colony preparing for winter by reducing their activity level, the bees' extended foraging activity can account for the high percentage of animals disappearing outside of the colony (73%), thus not making the transition into the winter cluster.

A third explanation could be different colony requirements. An observation hive with two combs was used in the experiment by Bock, while a Mini-Plus Nucleus hive was chosen for the actual experiment. Depending on the availability of resources in the hive it might have been necessary for the bees to forage more intensely, thus leading to shorter periods of life through all groups, as was the case in this study compared to the study by Bock.

Obviously, the above-optimally reared bees showed the highest total and individual activity/week in comparison to the other groups. The sub-optimally reared bees showed an increase in activity during their second week of life but stayed more or less stagnant over the rest of the experimental period, while the 35°C-bees showed a steady increase in activity until their fifth week.

Longer foraging trips performed by the above-optimal group could have two reasons. Groh and colleagues (Groh et al. 2004) found smaller number of microglomeruli (MG, synaptic complexes) in the olfactory input region (lip) of the honeybee mushroom body (MB) calyces of newly hatched and seven-day old bees, when the temperature during the pupal development differed by +/- 1 °C from the optimal 34.5°C. Since the mushroom body is thought to play an important role in learning and memory formation processes (Menzel, Erber, and Masuhr 1974; Hammer and Menzel 1995) it might be proposed that the association of floral odor with a reward in form of nectar

and pollen is in some way impaired in the 36°C-bees so that it takes them longer to identify the correct plants, which lead to extended foraging trips. On the other hand, longer foraging trips can be an indication that bees that experienced higher temperatures during the pupal phase develop in a different, faster way. Their earlier and higher activity compared to the 35°C- and 32°C-group can be interpreted as precocious foraging activity also observed by Becher and colleagues (Becher et al. 2009). Considering the time of year and the natural lack of nectar flow during this period, it can be assumed that the foraging perimeter increased rather quickly. Observing the activity and the time period spent outside the hive by the 35°C-bees, it becomes obvious that they seem to lag behind in both parameters by about a week. Furthermore, the significantly better performance of the bees reared at 36°C during a one-trial olfactory conditioning paradigm compared to bees reared at 32°C and 35°C (Tautz et al. 2003) could identify them as above-average foragers, explaining a possible earlier transition to outside duties.

The reduced longevity of the 32°C-group can be associated with the decrease of population by >25% during the first two weeks of life. This phenomenon of rapid decrease of experimental population in the sub-optimally reared group has also been observed by Tautz and colleagues. In addition, they reported impairments of the dance behavior by the bees reared at 32°C compared to the other groups when being trained to a food source of known location. They assumed that the orientation might be impaired leading to a loss of bees already during the orientation flight period. Usually the first two weeks of a bee’s life are used for orientation flights. These flights are needed in order to help the bees to make themselves familiar with the area surrounding the hive (Becker 1958; Capaldi and Dyer 1999; Capaldi et al. 2000). Inability to orientate themselves in the perimeter of the colony can serve as an explanation for a reduced return rate during periods of orientation flights. The data showed that from the 51 bees missing, two weeks after hatching, 40 (78.43%) were registered moving outward on their last registration. Nevertheless, the ultimate cause for the disappearance remains unclear, since it is also possible that they had drifted into other close-by colonies (the experiment was conducted in Würzburg) that were not monitored by RFID-readers.

VIII. Determination of sub-lethal insecticide effects on the foraging behavior of Apis mellifera using Radiofrequency identification (RFID)

VIII. 1. Summary

The development of insecticides requires valid risk assessment procedures to avoid causing harm to beneficial insects and especially to pollinators such as the honeybee *Apis mellifera*. In addition to testing according to current guidelines designed to detect bee mortality, tests are needed to determine possible sublethal effects interfering with the animal's vitality and behavioral performance. Several methods have been used to detect sublethal effects of different insecticides under laboratory conditions using PER-conditioning and -habituation. Furthermore, studies which require intensive visual observation have been conducted on the influence insecticides have on foraging activity and homing ability. An experimental design using radiofrequency identification (RFID) was used to monitor the influence of sublethal doses of insecticides on individual free-flying honeybee foragers without protracted visual observation or need of confining the hive in semi-field conditions. This allowed us to efficiently retrieve detailed information on flight parameters. At field-relevant doses for nectar and pollen no adverse effects were observed for either substance. The influence of different dosages of a tested substance, fed simultaneously to several groups of bees was compared. With this experimental approach effects of sublethal doses of the neonicotinoids imidacloprid (range: 0.15 – 6ng/bee) and clothianidin (range: 0.05 – 2ng/bee), and the organophosphate coumaphos (range: 1 - 5µg/bee) were monitored under field-like circumstances. All three substances led to significant impairments of foraging behavior, beginning at doses of 0.5ng/bee (clothianidin), 1.5ng/bee (imidacloprid), and 2µg/bee (coumaphos), during the first three hours after treatment with the effects of coumaphos lasting up to 48 hours after administration. These results confirm that the RFID-method is an effective way to record short-term alterations in foraging activity after insecticides have been administered once, orally, to individual bees. By investigating the effect of two different substance classes, new information on the

understanding of how honeybees are affected by non-lethal doses of insecticides is contributed.

VIII. 2. Introduction

VIII.2.1. Insecticides

VIII.2.1.1. History

The use of insecticides for insect control can be traced far back into the past. There is information that sulfur has already been used as a fumigant against pests (Shepard 1939; Ware and Whitacre 2004) during the time of Homer 1000 B.C.. In times of the Roman Empire, Pliny the Elder (A.D. 23-79) recorded most of the earlier insecticide uses, e.g. use of gall from a green lizard to protect apples from worms and rot, in his *Natural History*. Arsenic sulfides were used by the Chinese as early as 900 A.D. for insect control (Shepard 1939; Haller 1942). The first botanical insecticide, used as such, was nicotine. Since the end of the 17th century it was won from tobacco leaf decoctions for the use against aphids (Lapierre 2004). It was successfully isolated from the tobacco plant in 1828 (Posselt and Reimann 1828), and successfully synthesized at the beginning of the 20th century (Pictet and Rotschy 1904). Other insecticides occurring naturally were among others rotenone, pyrethrum (served as lead for the synthesis of pyrethroids), and hellebore (as reviewed in Soloway 1976).

The beginning of World War II was also the beginning of the era of synthetic insecticides, starting with the organochlorine DDT (**d**ichloro**d**iphenyl**t**richloroethane). First synthesized in 1874 (Zeidler 1874), its insecticidal properties against arthropods were not discovered until 1939 (Paul Müller, Nobel Prize for medicine and physiology in 1948). Since then, synthetic organic insecticides have largely replaced botanical and inorganic insecticides in form of organophosphates, methylcarbamates, and pyrethroids (Tomizawa & Casida 2005). Over the last four decades a new class of synthetic insecticides has become a commonly used alternative for crop protection: The neonicotinoids.

The first prototype, nithiazine, was first synthesized in the late 1970s by Solloway and colleagues (Soloway et al. 1978), but due to its low stability, nithiazine could not be used for practical application. In 1985, the economically most successful neonicotinoid, imidacloprid, was synthesized for the first time by Kagabu and

colleagues and six years after this discovery in 1991 imidacloprid was introduced to the market for the first time. Known today under the names of acetamiprid, clothianidin, dinotefuran, nitepyram, thiacloprid, thiamethoxam, neonicotinoids are used mainly for crop protection against piercing and sucking pests, but also for flea control on cats and dogs (Tomizawa and Casida 2005). Their high selectivity for insect nAChRs compared to mammalian nAChRs, their high effectiveness and their systemic properties provide them with advantages over other insecticidal compounds.

VIII.2.1.2. Neonicotinoids

Mode of action

Even though the neonicotinoids imply the word nicotine, they originated from a screening of novel synthetic chemicals. Nevertheless they resemble nicotine in their mode of action and partially in their structure (Tomizawa and Casida 2003). Both nicotine and neonicotinoids are agonists of the nicotinic acetyl choline receptor (nAChR) and similar to the endogenous neurotransmitter actylcholine (ACh) both bind to the nAChR, either at the same binding site or at a close by area (Sattelle et al. 1989; Bai et al. 1991; Nishimura et al. 1994), leading to an activation of the ion channel. Neonicotinoids act as partial agonists or even “super agonists” on insect nAChR. Imidacloprid was identified as a partial agonist of nAChRs from honeybee Kenyon cells since it elicited only 36% of ACh-induced currents (Déglise, B. Grünewald, and M. Gauthier 2002). Ihara et al. (2006) showed that imidacloprid and clothianidin are partial agonists of nAChRs of the terminal abdominal ganglion of the American cockroach *Periplaneta Americana*. Again in 2006, Brown et al. demonstrated a super efficacy action for clothianidin since it elicited currents up to 56% larger than those evoked by ACh in cholinergic neurons of 3rd instar *Drosophila* larvae.

Selectivity

It is proposed that different amino acid combinations of the insect and the mammalian nAChR lead to selectivity differences. While nicotine is protonated at a physiological pH (pK_a= 7.90 - 8.18) (Yamamoto 1965; Chamberlain, A. A. Evans, and Bromilow 1996) neonicotinoids, like imidacloprid (pK_a= 1.56), are not (Chamberlain, A. A. Evans, and Bromilow 1996). Instead of an easily protonated nitrogen group,

most neonicotinoids carry an electronegative nitro or cyano group. It is presumed that this negatively charged pharmacophore interacts with cationic amino acids (e.g. lysine, arginine, histidine) of a subsite of the neonicotinoid binding region (Tomizawa et al. 2000; Tomizawa et al. 2003).

Specificity and Effectivity

The high affinity of neonicotinoids to the insect nAChRs can be demonstrated by comparing the inhibitory concentration 50% (IC_{50}) needed to displace [^{3}H]imidacloprid at the [^{3}H]imidacloprid-binding site on the insect nAChR. This receptor site was identified in 1992 by using the radioligand [^{3}H]imidacloprid (Latli & Casida 1992). The IC_{50} value for imidacloprid (4.6 nM) and clothianidin (2.2 nM) binding at the *Drosophila* nAChR is 870 to 1818 fold lower than the IC_{50} for nicotine (4000 nM) at the same receptor (Tomizawa et al. 2003; Tomizawa and Casida 2005a).

Additionally, the effective response concentration 50% (EC_{50}) which is determined for drugs, toxins and antibodies, is usually found to be lower than the EC_{50} for nicotine or ACh. The EC_{50} values found for the most studied neonicotinoid imidacloprid range around the nanomolar/micromolar threshold (0.3 - 25µM) probably depending on type of neurons used (Brown et al. 2006; Schmuck et al. 2001; Nauen et al. 2001; Buckingham et al. 1997; Barbara et al. 2007; Déglise et al. 2002). The EC_{50} values for clothianidin found for Larvae of the tobacco budworm (*Heliothis virescens*), for cholinergic neurons from *Drosophila*, and for dorsal unpaired median (DUM) neurons from the American cockroach was 0.309 µM 1.8µM, and 1.7µM (Nauen et al. 2003; Brown et al. 2006; Thany 2009). The respective values for nicotine and acetylcholine, considering the same type of neurons, range from 3.76 – 7.9µM and 6.3 – 19µM (Barbara et al. 2007; Brown et al. 2006; Barbara et al. 2005).

The systemic properties of neonicotinods combined with the application to seeds allow protection from the earliest stage of the plant. Translocated along the xylem, the insecticide is distributed throughout the young plant to protect it against different piercing and sucking pests. Experiments using radiolabelled [^{14}C]imidacloprid on the seeds of the sunflower (*Helianthus annuus* L.) have shown that the plant absorbed only about 10% of the imidacloprid used on the seed (Laurent and Rathahao 2003). Furthermore it was shown that the concentrations of imidacloprid were 20 fold higher

in the first leaves compared to the upper leaves. Residues of radiolabelled imidacloprid were detected in pollen (3.9µg/kg) and nectar (1.9µg/kg) of sunflowers (Schmuck et al. 2001). Laurent & Rathahao (2003) who used 1mg imidacloprid/seed (30% more than recommended) found residues of 13µg/kg in pollen. Chauzat et al. (2006) found imidacloprid residues (avg. 1.2µ/kg; max. 5.7µg/kg) in 49.4% of pollen load samples collected from apiaries all over France. Clothianidin ([^{14}C]clothianidin) was also found in samples of pollen and nectar collected from summer rape, sunflower and corn with residue levels of <10µg/kg (Schmuck & Keppler 2003). These dosages of neonicotinoid substances found in the nectar and pollen are usually too low to kill non target insects, such as pollinators but little is known about possible sublethal side effects after exposure.

VIII.2.1.3. Varroacides

The exposure of honeybees to insecticides is not exclusively limited to the field. The occurrence of the parasitic mite *Varroa destructor* (Anderson and Trueman 2000) in colonies of the European honeybee (first appearance in Germany in 1977, Ruttner & W. Ritter 1980), and the fact that there has been no extended parasite-host co-evolution compared to the original host, the Eastern honeybee *Apis ceranae* (Rath 1992), made it necessary to apply insecticides to the hive in order to control the mite population and spread, thereby preventing the collapse of the infested colony. It is proposed that without proper colony treatment, especially in temperate climates, colonies of *A. mellifera* will be damaged to the extent of collapse within a few years (Boecking & E. Genersch 2008; Rademacher & Imdorf 2004, reviewed in Rosenkranz et al. 2010).

Since the first occurrence of *Varroa destructor* a wide range of chemical substances has been used for mite control. Pyrethroids like tau-fluvalinate and flumethrin proved to be very effective against the mite without any considerable side effects on the bees. Apparently, strains of *V. destructor* started to show resistance to fluvalinate (Milani 1999; Milani 1995) resulting in a cross resistance to other pyrethroids (acrinathrin, flumethrin, reviewed in Rosenkranz et al. 2010).

Another substance commonly used for *Varroa* control is the organophosphate coumaphos (Ritter 1985), active ingredient of products like Perizin®, Checkmite®, or Asuntol®. Belonging to the organophosphates, it serves as an inhibitor of the acetylcholineesterase (AChE), phosphorylating the active center (O'Brien 1963;

O'Brien 1966) and thereby inactivating the hydrolysis of acetylcholine (ACh) into acetate and choline allowing ACh to accumulate at cholinergic synapses (as described in Purves, 2008). It has been found that phosphorylated enzymes can lose an alkoxy-group over the next 24 - 48h, a process referred to as "aging" (Vale 1998 as reviewed in Kwong 2002), ultimately leading to irreversible damage to the enzyme. Powerful organophosphorous agents like sarin own their action to rapid rate of "aging" (P. Taylor 2001,22:).

The LD_{50} of Coumaphos for honeybees has been found to vary between young and older bees. Bees that were three days old had an LD_{50} of 6.04µg/bee while bees that were eight and 14 days old had an LD_{50} of 2.99µg/bee and 3.10µg/bee, respectively (van Buren et al. 1992). Coumaphos belongs to the substances most commonly found in recent residue analysis studies. Its lipophilic properties not only allow coumaphos to easily travel into the wax of the combs but also lead to contamination of bee products (honey and pollen) that are stored in wax-contaminated cells (reviewed in Wallner 1999; Kochansky et al. 2001; Blasco et al. 2003; Chauzat et al. 2006; Martel et al. 2007; Chauzat & Faucon 2007; Elke Genersch et al. 2010). The residues detected in wax, pollen, and honey differ by several orders of magnitude but were not found to be in a range, according to the LD_{50}, that would prove lethal for honeybees. Again, as for the residues of neonicotinoids, the questions remains of how low doses of a varroacide like coumaphos, might affect the behavior of honeybees if it does not kill them.

VIII.2.2. Sub-lethal Effects and their Detection

Sublethal effects have been described as effects on physiology and behavior of an individual that has been exposed to a pesticide iwithout directly causing death (Desneux et al. 2007). Desneux et al. defined a sublethal dose as a dose "inducing no apparent mortality to the experimental population". For honeybees exposure to sublethal insecticide doses can have an influence on learning ability, orientation, foraging, or brood care (Thompson and Maus 2007).

Standard risk assessment procedures that have been developed to evaluate the effects of plant protection products on non-target organisms aim at detecting mortality. Since honeybees in their role as pollinators interact with plants that are targeted by insecticide application, standard guidelines have been developed to

assess the risk of these substances. These tests include toxicity evaluations on adult bees by cage-, tunnel-, and field experiments (EPPO 1992; OECD 1998; OEPP/EPPO 2003). Residual toxicity is considered to be less important, due to the fact that these guidelines deem the main exposure way to be by spraying application. Through their systemic properties, insecticides like organophosphates and neonicotinoids, seed dressing has become a major practice for plant protection. It effectively reduces the amount of insecticides used on agricultural crop land by up to 99% (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, BVL, Germany). Furthermore, seed dressing is supposed to reduce health risks by minimizing the contact of environment with the active ingredients. Nevertheless, the environment is exposed to these substances. Considering this, the effects of sublethal insecticide doses should not be neglected in standard risk assessment procedures. There are two main exposure routes for non target organisms, also considered in standard risk assessment procedures: contact and oral ingestion. Contact exposure can occur during the sowing process of dressed seeds. Parts of the active ingredient might peel off the seed and lead to dust drifts through exhaust air of the drilling machines resulting in contamination of insects and flowering plants in the nearby area. Honeybees can be exposed by different ways when considering oral ingestion. These include nectar, pollen, and guttation water. The latter, an excretion of xylem water at the leaf margins, was recently discovered to hold high residues of neonicotinic substances (imidacloprid, clothianidin, thiamethoxam) when collected from treated corn seedlings (*Zea mays* L.) (Girolami et al. 2009). Until now, though, it still remains unclear if water foragers collect guttation water from seed dressed plants and, if they do, how these drops affect the bees.

Several methods have been developed to study the influence of non lethal doses of insecticides on honeybees, bumblebees and solitary bees. The most prominent is a paradigm for olfactory conditioning, the proboscis extension reflex (PER) conditioning (Frings 1944; Takeda 1961). This simulates the conditioning process of memorizing a floral cue, e.g. smell, and associating it with the reward, in this case nectar and pollen (Menzel & Müller 1996). A classically conditioned response uses the ability of the honeybee to associate these two stimuli, a conditioned stimulus (CS) being the presentation of an odor (substitute for floral odor) and an unconditioned stimulus (US) being sugar water (substitute for nectar) presented almost simultaneously to the antennae. Latter stimulus triggers an extension of the proboscis. After several

training bouts the tested individuals have learned to associate the CS with the US and extend their proboscis when presented only with the CS. This model is used to assess the impact of sublethal doses of different insecticides on e.g. olfactory learning. Taylor et al. (1987) found a reduced initial learning response and a reduced final degree of learning when bees were treated with six different pyrethroids. Stone et al. (1997) reported that the hydrocarbon acaricide dicofol, an analog of DDT, reduces the acquisition and the extinction. Another acaricide, the organophosphate coumaphos, was reported to have no significant effects on learning and memory when applied topically or injected intracranially in sublethal doses (Weick and Thorn 2002). Fipronil, a phenylpyrazole known to block to GABA (γ-Aminobutyric acid) receptors of glutamate gated chloride channels lowered sucrose sensitivity and learning ability after topical but not after oral application (Abdessalam Kacimi El Hassani, Matthieu Dacher, et al. 2005). Many PER studies have focused on the detection of sublethal effects elicited by neonicotinoids. The most frequently studied neonicotinoid imidacloprid was found to facilitate habituation of the PER at doses of 1.25ng when applied topically (Lambin et al. 2001). Negative effects on learning performance were found at levels of 12 µg/kg for chronical and 12 ng/bee for one-time administration (Decourtye, Lacassie, and Pham-Delègue 2003; Decourtye et al. 2004).

In field experiments the influence of sublethal insecticide doses is investigated by analyzing the foraging behavior at an artificial feeder containing sugar water as a nectar substitute and/or by analyzing the homing ability of the treated bees (Vandame et al. 1995; Bortolotti et al. 2003; Colin et al. 2004; Yang et al. 2008), but the observations are time-consuming and the information provided is limited. Therefore, I wanted to use a feeder task combined with RFID labeling (Streit et al. 2003; Sumner et al. 2007; Moreau et al. 2010) to obtain detailed information on foraging behavior with little effort and at reasonable cost. Independently from this study, Decourtye and colleagues (Decourtye et al. 2011) simultaneously developed a similar design which, like the approach in this study, was based on two sets of separate direction-sensitive reading devices positioned in front of the hive entrance and in front of a compartment containing an artificial feeder. They found that the phenylpyrazol insecticide fipronil prolonged the homing flight of honeybees for up to three days. While Decourtye focused on the effects of fipronil on longevity, as well as the number and duration of the homing flight, this investigation focused on the effects

of the neonicotinoids imidacloprid, clothianidin, and the organophosphate coumaphos foraging behavior. Alterations in foraging behavior could be observed in the changes of parameters like foraging activity, the total duration of foraging trips, which in addition were split up into flight time to the feeder, duration of stay at the feeder, and flight time back to the hive, as well as the time interval a bee spent inside the hive between foraging trips were the focus of this study.

Since low doses of insecticides are known to disrupt learning processes necessary for the correct association of olfactory or visual stimuli offered by a plant with a reward in form of nectar and pollen, they also might cause visually non-observable effects on motoractivity and orientation ability, leading to reduced foraging activity or prolonged foraging flights.

VIII.2.3. Foraging behavior of *Apis mellifera*

Foraging for resources like pollen and nectar belongs to the most important tasks of a honeybee in order to secure the survival of the colony. Minimum annual requirements of a colony for pollen were found to be between 15 and 30 kg, but colonies can collect up to 55kg (Eckert 1942; Hirschfelder 1951; Louveaux 1958; Seeley 1985). The weight of the pollen loads carried home by individual foragers, were found to be ranging between 8 and 29 mg (Gillette 1897; Park 1922; Parker 1926) resulting in an average of about 15mg (as reviewed in Winston, 1987) and a need of about one million foraging trips to collect the minimum pollen requirements.

The results found for the annual honey requirements range even higher between 60 and 80 kg (Weipple 1928; Rosov 1944; Seeley 1985). The weight of a single nectar load can vary between 12 and 70 mg (Lundie 1925; Parker 1926; Fukuda, Moriya, and Sekiguchi 1969; Combs 1972) resulting in an average of about 32 mg nectar containing about 16mg of sugar (as reviewed in Winston, 1987) and about four million foraging trips for collection of the minimum honey requirement.

The task of collecting these amounts of pollen and nectar requires a large scale search of the area surrounding the hive. A detailed dance analysis to detect the distance of foraging sites in a forested area was conducted by Visscher and Seeley (Visscher & T D Seeley 1982, after the example of Knaffl 1953), and revealed that 95% of all foraging flights remain within a 6 km radius around the hive, with a median

at 1.6 km, a mean at 2.2 km, and a maximum of 10.9 km. The 6 km radius equals an enormous area of around 100 km^2 which is scanned for resources.

Studies by Parker (Parker 1926) and Free (Free 1960) observed task specialization in foragers. They found that 58% of the bees were pure nectar foragers, 25% pure pollen foragers, and 17% were collecting both nectar and pollen during their forager life. Similar to this constancy of task, foragers show another type of constancy: flower constancy. Bees tend to forage from the same type of flower as long as it provides resources, or until a more favorable source becomes available. Flower constancy is easily detectable by examination of the pollen loads brought back to the colony (Betts 1935; Free 1963).

Mechanisms to optimize the foraging efficiency of this area are coordinated inside the colony which serves as an information center. Winston (Winston 1987) divided these mechanisms into two major groups: (1) Mechanisms that obtain information about the colony needs, and (2) mechanisms that allocate foraging tasks.

(1) Reports have shown how the lack or the surplus of pollen (Lindauer 1952; Free 1967; R. J. Barker 1971; Free and Williams 1971; Moeller 1972; Fewell and Winston 1992) led to a respective increase or decrease in the number of pollen foragers. It was shown that the number of pollen foragers also varies with the amount of brood present in the colony (Filmer 1932; Free 1967; Todd and Reed 1970; Dreller, Page Jr, and Fondrk 1999; Fewell and Winston 1992). Though not as thoroughly investigated it is presumed that factors like queen pheromone, presence of worker larvae, and empty combs have an influence on nectar foraging (Jaycox 1970a; Jaycox 1970b; Rinderer 1981).

(2) Knowing about the requirements of the colony, some of the bees now need to discover foraging sites in the surrounding area (scouts) and communicate the location and quality of the resource to their hive mates (recruits) for exploitation. Seeley (Seeley 1983) defined scouts as bees returning to the colony with pollen and/or nectar without having followed a dance and he determined that a proportion of 5 - 35% (variability due to availability of resources) of the total number of foragers are performing scout duties. After returning to the colony, scouts provide information about the foraging source by offering samples of collected nectar, by the vigor and direction of their waggle dance, or by floral odors which they picked up at the foraging site. Thus, recruited workers can exploit the closest and most attractive resources surrounding the hive.

Interestingly, nectar foragers do not always load the maximal amount of nectar into their crop (von Frisch 1934). There are two theories trying to explain this potential inefficiency. Neukirch (Neukirch 1982) proposed that foragers can only fly a limited distance during their lifetime. This led to the idea that workers might apportion their expenditures of energy (Schmid-Hempel, Kacelnik, and Houston 1985). They maximize the ratio weight of nectar load per flight distance, thereby maximizing the energetic gain per unit of energy spent. Núñez (Núñez 1982) has presented another approach. He presumed that foragers return to the hive with a partially filled crop whenever the resource quality is suboptimal. Thus, they need less time to fill their crop and can return to the colony more rapidly where other hive mates might advertise a more profitable foraging source. Collecting full nectar loads could lead to a loss of information.

The individual bee does not only collect information inside the hive but also on its foraging trips. In order to provide accurate information about the direction and the distance of the foraging source during dances, honeybees use features like polarized light vision (von Frisch 1949), sun compass (Lindauer, 1954; von Frisch, 1967), and the image motion while flying, known as the optic flow (M. V. Srinivasan et al. 1996; Esch et al. 2001; Si, M V Srinivasan, and S Zhang 2003). Furthermore, they can use orientation cues like memorized positions of landmarks (von Frisch & Lindauer 1954; von Frisch 1967; Dyer & Gould 1981; T S Collett 1993; T S Collett & Baron 1994; M. Collett et al. 2002) on the way to the foraging destination, and floral cues like odor, color, shape, and UV-patterns at the foraging destination.

Additionally, there is the idea that bees can navigate according to a cognitive map of a familiar territory. According to Bennett (Bennett 1996), there are two main definitions of a cognitive map in animals. Tolman (E. C. Tolman 1948) and O'Keefe & Nadel (O'Keefe and Nadel 1978) defined a cognitive map as a memory of landmarks in a familiar environment which allows novel short cutting between two points to occur. Gallistel (Gallistel 1990) offered a different view of a cognitive map. According to his view, a cognitive map of the environment is a representation of macroscopic geometric relations among surfaces in the nervous system of the animal, used to plan movement in the environment. Bennett argues though, that there are more simple explanations for short-cutting which have to be excluded before considering the existence of a cognitive map. Novel short cuts have to be truly novel, pathintegration (H Mittelstaedt and M L Mittelstaedt 1973; M L Mittelstaedt and H

Mittelstaedt 1980) has to be excluded, and it has to be made sure that familiar landmarks are not recognized from different angles. Using harmonic radar to follow complete flight trajectories, Menzel and colleagues (Menzel et al. 2005) made a strong case for the existence of a cognitive map in honeybees, observing short cutting after release at an arbitrary location in the surrounding environment of the hive (orientation flight range) and ability to choose between different goals (hive or artificial feeder). This led them to the conclusion that the question is not “whether there is a map-like spatial memory but rather what structure this map has and how it is used” (p. 3045).

The question that arises is how non-lethal amounts of insecticides like neonicotinoids and varroacides might affect foraging behavior. Contact with low insecticide doses could impair accuracy and effectiveness of the waggle dance, as communicated by Kirchner on the 46th meeting of the “Association of Institutes for Bee Research“(1999). Inaccuracy of the waggle dance has already been shown in studies concerning different stresses aside from insecticides e.g. bees being raised by suboptimal temperatures (Tautz et al. 2003) as well as for sleep deprived bees (B. A. Klein et al. 2010).

VIII. 3. Material & Methods

VIII. 3.1 Bee material

For our experiments, an *Apis mellifera carnica* breeder line housed in three nucleus bee hives (Mini-Plus, Bienenzuchtbedarf Heinrich Holtermann GmbH & Co KG, Brockel, Germany) was used. Each hive contained 6 Mini combs (approx. 248 x 159 mm). The hives were aligned in a row 1-2 m apart. Each hive entrance was marked by black geometrical shapes (triangle, circle, and square) on a white background to provide visual guidance for the returning bees.

Figure 19: Collection of flight bees with a handheld vacuum cleaner in the perimeter of the blocked hive entrance.

VIII. 3.2. Toxicity Tests

Depending on the level of knowledge about the substances tested in this study, cage-tests (**Fig. 20**) were conducted to assess the toxicity. Since the first substance the neonicotinoid imidacloprid has been subject of numerous studies, it was decided to use 3ng/bee for testing in 2008. When it was possible to test several treatment groups simultaneously in 2009, the maximum threshold was raised to 6ng/bee (see also chapter V.3.5.). These doses were chosen in regard of the lowest LD_{50} reported in the literature (3,7ng/bee, Schmuck et al. 2001). The second substance, coumaphos, has also been the subject of several studies. The range of the LD_{50} for coumaphos is assumed to be between 3 and 6µg/bee. According to these values, the doses for coumaphos were chosen between 1µg/bee and 5µg/bee. In order to test for an appropriate threshold for the third substance, the neonicotinoid clothianidin, four cage-tests were conducted on the 25.05., 02.06., 08.06., and 12.06.2009.

Figure 20: Stainless steel cage with feeding tube used in the toxicity cage tests.

Bees of unknown age were caught at the hive entrance by blocking the flight hole and collecting the returning bees that landed in the area around the sealed hive

entrance with a handheld, cordless vacuum cleaner using a custom made collecting

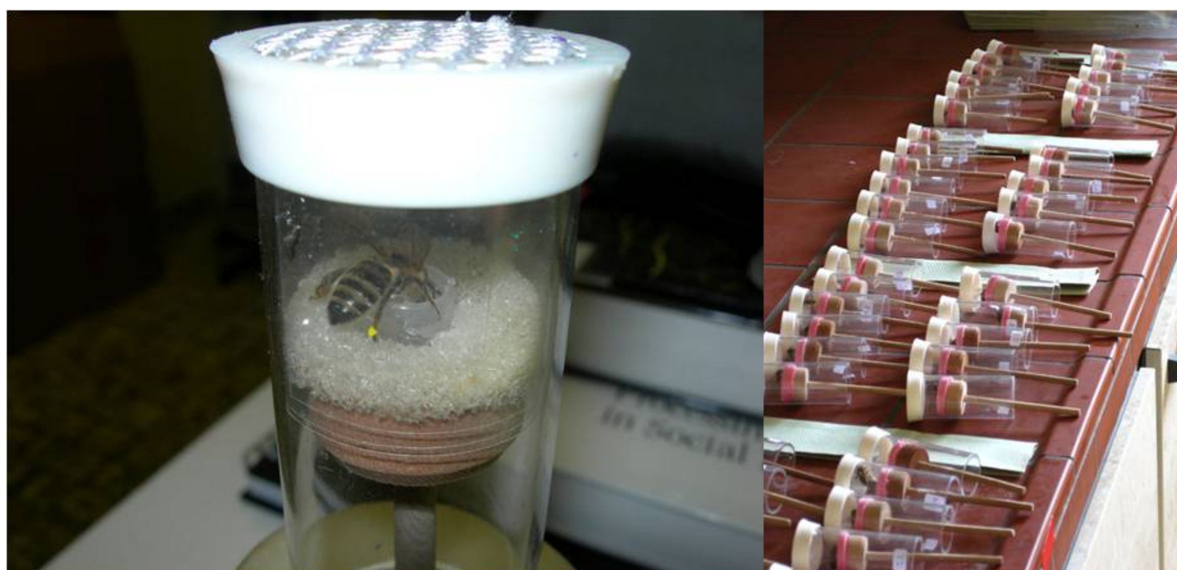

Figure 21: Individual feeding of known amounts of a certain substance in known volumes of sugar solution in a queen marking tube.

adapter (**Fig. 19**). Subsequently, the collected bees were transferred into a communal cage and starved for 2 hours in darkness (in an incubator at 20°C and a humidity of 60-70%). To analyze influences of insecticides on individual bees, the objective of the RFID studies, an individual feeding technique instead of the standard bulk feeding procedure during honeybee toxicity tests (OECD Testing Guideline 213) had to be developed. Thus, following the starvation period, the bees were taken from the cage one after the other and treated orally with 10µl of sugar solution (2M) in which a certain dose of clothianidin was dissolved. For this process queen marking tubes (Fig., Carl-Fritz-Imkereifachhandel, Mellrichstadt, Germany) similar to those used for RFID-labeling were used. The sugar solution was offered in a cap, removed from a 1.5 ml Rotilabo® micro centrifuge tube (Carl Roth GmbH, Karlsruhe, Germany), embedded in the foam plastic of the plunger, thereby serving as a small feeding trough (**Fig. 21**). In addition to a control solution, containing an equivalent of the solvent (acetone) used to pre-dissolve clothianidin, four different doses were administered in each experiment. The doses used were 0.15ng, 1.5ng, 3ng, and 6ng/bee which were equivalent to 10µl of the following concentrations: 0.06µM, 0.6µM, 1.2µM, and 2.4µM (for detailed information concerning the preparation of the solutions see Chapter VIII.3.5. “Administration of Insecticides”). Each cage contained 10 +/-1 bees (depending on the willingness of the bees to consume the offered solution). Bees that did not or not fully consume the offered solution were discarded

from the experiment. After consumption the bees were introduced into a stainless steel cage (**Fig. 20**) where they were provided with 50% honey-solution (w/v) *ad libitum*. In the initial two cage experiments (25.05., 02.06.2009) a number of two replica cages per treatment group was used. This number was raised to three replicas per group in the latter two experiments (08.06., 12.06.2009). The cages were stored in a dark room at room temperature (~20°C) and a humidity of ~50-60%. Mortality and signs of abnormal behavior were recorded 2h, 4h, 24h, and 48h after treatment. Bees were declared dead when no signs of movement were observed anymore. Dead bees were removed from the cages immediately. The LD_{50} (24h and 48h) within 95% confidence limit was calculated by Probit-Analysis (SPSS Statistics 17, IBM Corporation, Somers, NY).

VIII. 3.3. Experimental design

Between 2008 and 2010 twenty experiments were conducted to analyze the effects of imidacloprid, coumaphos, and clothianidin on honeybee foraging behavior. Those divide as follows: three experiments with imidacloprid, eight experiments with coumaphos, and nine experiments with clothianidin. All experiments took place at a research facility distanced 370 meters east of the “Institut für Bienenkunde” in Oberursel. All time specifications mentioned in the following are related to dry (sunny or overcast), warm (T ≥ 20°C) weather.

VIII. 3.3.1. Foraging setup

The behavioral paradigm to analyze foraging behavior was to train the bees to forage at an artificial food source and monitor their performance. Therefore, a feeder (filled with 2 M sucrose solution) was placed either close to (7 meters) or further away (370 meters) from the hive entrance. For the short-distance experiments, departing worker bees of unknown age were caught at the hive entrance, using sample bottles with snap caps (70 x 25 mm, 20 ml, neoLab, Heidelberg, Germany) and carried to the feeder. There, the bees were released by placing the opening of the sample bottle as closely as possible to the feeder so that the bees could collect sugar solution if it seemed attractive enough to them. To offer the bees additional visual and olfactory cues to be associated with the food source, a square-shaped 75x75 mm yellow wax

patch from comb foundations was placed beneath the feeder. Bees returning to the feeder on their own accord were color-marked on the abdomen. The number of bees that were color-marked on a given day ranged between 90 and 100 individuals.
At the end of August 2010, a mid-distance experiment was conducted where bees were trained to find the feeder in a distance of 370 meters. Therefore the feeder was moved stepwise away from the hive until the final destination was reached. To get the bees used to the feeder it was placed right in front of the flight hole upon a yellow bucket which served as a landmark. All bees foraging from the feeder were marked with a white dot (Edding® 750 paint marker, Edding International GmbH, Ahrensburg, Germany) on the abdominal region which was continued throughout the next training steps until 100 individuals had been marked. After an hour, the feeder was distanced 15 cm further away from its original position. This was repeated twice over the next two hours. During the next three hours, the feeder was distanced ½ meter (after 45 minutes), 1 meter (after 45 minutes), two meters (after 30 minutes), five meters (after 30 minutes) and ten meters (after 30 minutes) from the previous position, respectively. At a distance of 7 meters, the location of the short-distance experiments, the bees were trained to enter and leave a self-designed feeder compartment (see also Chapter VIII. 3.3.2.2) which was needed later on for automatic registration. The housing development and vegetation between the hive and the feeder destination did not always allow the relocation of the feeder in a straight line during the training (**Fig. 22**). The interval between straight-line relocations of 10 meters was 15 minutes. At the two more or less 90°-direction changes along the training route relocation distance was reduced to five meters.

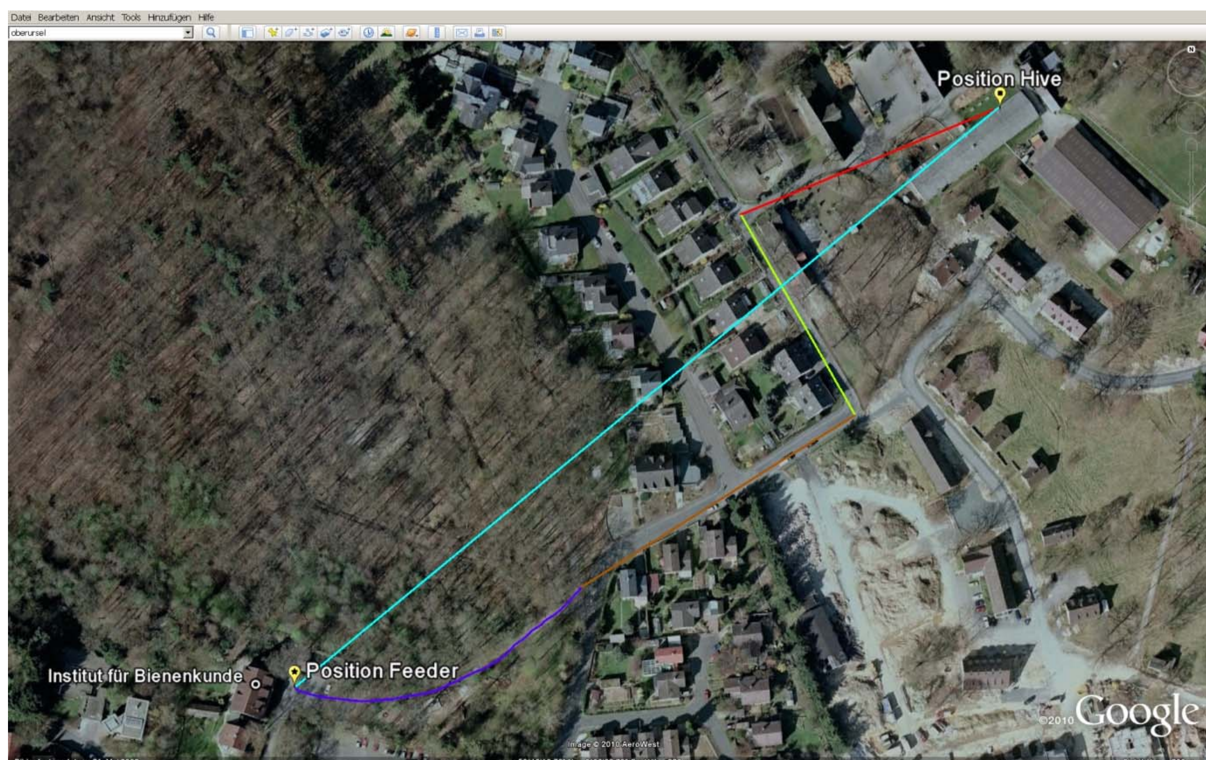

Figure 22: Maximum distance between hive and Feeder (370m beeline). Red (112 meter), yellow (93 meter), and brown (128) lines mark the routes where straight-line feeder relocation was possible. The purple path (132 meters) marks the route along a forest path towards the Institut für Bienenkunde. (Screenshot taken from Google Earth).

The time duration until the next feeder relocation after the direction change was prolonged to a total of 20-25 minutes, depending on the ability of the bees to detect the new position. The last stage of training led along a slightly arching forest path towards the feeder destination. Due to this difficult terrain the relocation interval was prolonged from 15 to 20 minutes. The complete training procedure lasted between three and five days depending on weather conditions. At the beginning of each new day the feeder had been set up at exactly the same position where it was removed the day before.

VIII. 3.3.2 Monitoring by RFID technology

VIII. 3.3.2.1 Manual detection at the Feedersite (2008)

Figure 23: Monitoring of every visit of a tagged bee at the feeder site with a hand held USB-Reader-Pen.

During the year 2008, the foraging activity (number of feeder visits during an observation period) at the feeder was monitored by the experimenter using a handheld Reader-Pen (iID® PEN mini USB, microsensys, Erfurt, Germany) combined with the custom software from miscrosensys which displayed the TAG ID

on a notebook display (**Fig. 23**). The time of every arrival of a tagged bee was noted and counted as a visit. In front of the hive the activity of the bees and the total time needed for a single foraging trip was registered by the scanners. During the experiments in the spring and summer of 2008 three parameters of foraging behavior were detected: 1) The frequency of feeder visits during a defined observation period, 2) the time duration of a foraging trip, and 3) the interval spent inside the hive between foraging trips.

VIII. 3.3.2.2 Automatic detection at the Feedersite (2009/10)

In order to add an exact analysis of other interesting parameters such as flight time to the feeder, time duration of the feeder stay, and duration of the homing flight a feeder compartment made from acrylic Plexiglas® (dimensions: 229 x 165 x 55 mm) was designed (**Fig. 24**). In order to forage from the feeder, each bee had to enter the compartment through an identical tunnel-system, as used at the hive entrances. Two readers mounted on tunnels registered every passage of an RFID tagged bee. Combining this data with the data from the readers at the hive entrance allowed a detailed analysis of six different parameters of foraging behavior (**Fig. 25**).

Figure 24: Feeder chamber enabling automatic registration of RFID tagged bees entering and leaving the food source.

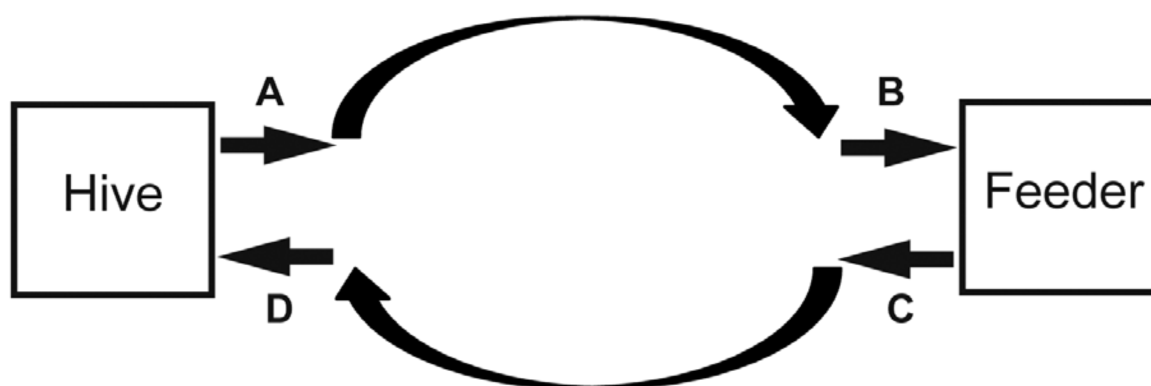

Figure 25: Measuring of multiple parameters of a foraging trip with readers positioned at both the hive and the feeder. In order to leave the hive for a foraging flight, a tagged bee had to pass the reading devices at the hive entrance in an outward bound direction (A). After departure, they had two options: Either forage from the artificial feeder or choose a random honey flow source (not shown by arrows) around the perimeter of the hive. When the tagged bees chose to forage from the feeder, they had to pass the reading devices monitoring the feeder compartment entrance (B). The frequency of visitation to the feeder was therefore characterized as the number of sequences of A followed by B. An additional parameter analyzed by this sequence was the time that elapsed between leaving the hive until entering the feeder compartment, which was described as “flight time to the feeder”. In order to leave the feeder compartment after a foraging stay, the tagged bees needed to pass the reading devices in front of the feeder in an outbound direction (C). From the sequence B followed by C we were able to calculate the time duration a bee spent at the feeder. The final step was the homing to the hive, where the tagged bee had to pass the reading devices in front of the hive entrance heading inward (D). The parameter described as “homing flight” can be calculated by the sequence of C followed by D. The overall time needed for a foraging flight is determined by the time the tagged bees left (A) and the time they returned to the hive (D). If the bees decided to forage from a different food source, only an overall time could be calculated. Finally, we could measure the period a bee spent inside the hive between two foraging flights by analysing the time of D and comparing it with the time of the subsequent departure from the hive.

VIII. 3.4. Feeder Compartment Training Procedure

Unlike the registration tunnels in front of the beehive, which the bees learned to pass of their own accord when leaving and entering the colonies, a stepwise training was required to guide the foragers to the feeder within the aforementioned feeder compartment. The compartment consisted of three separate parts which were plugged together to house the feeder. The parts were described as: i) base structure forming the floor and the surrounding walls, ii) lid which formed the ceiling, and iii) tunnel which formed the entrance to the compartment (**Fig. 26**). After the bees had been trained to forage from the freely accessible feeder, as mentioned above, the feeder on top of the wax patch was placed inside the base structure of the feeder compartment for about 30 minutes. The second step was to place the lid on top of the compartment leaving only the opening for the tunnel in the surrounding walls.

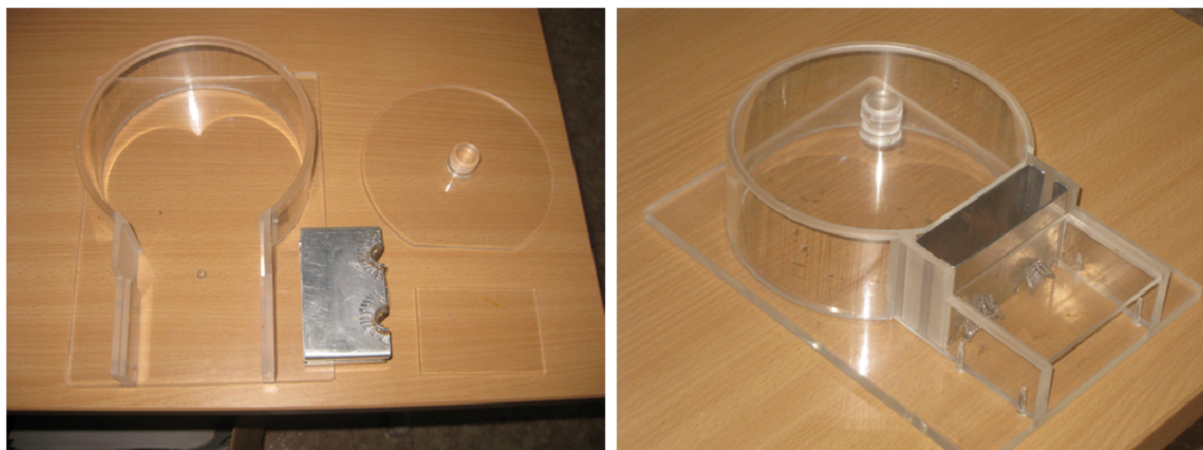

Figure 26: left: Feeder compartment parts, right: Feedercompartment assembled.

Thirty minutes later, when the bees had adjusted to the lid, a tunnel with wider tubes than the final registration tunnel was inserted, closing the compartment completely except for the two tunnel openings. Small wax patches (10 x 10 cm) similar to the one underneath the feeder were placed in front of the tunnel entrances. In addition a sugar trail containing the same concentration as the feeder filling was laid along the tunnel tubes aiding the bees in detecting and entering the tunnels. The sugar trail was constantly renewed until several foragers found their way inside the feeder compartment. These "pioneers" helped other foragers to enter the compartment. Since bees are positively phototactic, when they leave a feeding site (Menzel and Greggers 1985), the transparent compartment was covered in a way that the only light the bees could perceive came from the tunnel openings. After an hour the tunnels were switched to the original registration tunnels (see also **Fig. 2** in Chapter IV.1.). Subsequently, the cover was removed for short intervals until the bees had learned to leave the compartment through the tunnels without the cover attached. Finally, the wax marks at the tunnel entrances and underneath the feeder were removed to avoid excessive recruitment during the experiments.

VIII. 3.5. Administration of Insecticides

I was interested to see if the acute oral administration of certain insecticides altered the foraging parameters described above (see also VIII.3.3.2.2). For the tests imidacloprid (MW: 255,7 g/mol, powder form, Bayer AG, Leverkusen, Germany), clothianidin (MW: 249,7 g/mol, powder form, Sigma-Aldrich, St. Louis, Missouri, USA) and coumaphos (MW: 362,8 g/mol, in form of Perizin® 32 mg/ml, Bayer AG,

Leverkusen, Germany) were used. All three substances were applied orally. For the experiments conducted in 2008, which included testing imidacloprid and coumaphos, substances were dissolved in 33% honey solution. The idea was to give bees an additional olfactory cue, thereby shortening the duration of time needed to find and imbibe the spiked solution. In the experiments conducted in 2009 and 2010 the substances were either administered in a 2 M sucrose solution (imidacloprid and clothianidin) or 33% (w/v) sucrose solution (coumaphos).

The solubility of both neonicotinoids in water (imidacloprid: 0.51 g/L; clothianidin: 0.327 g/L) is rather poor. Thus, it was necessary to pre-dissolve 10 mg of both substances with 1 mL of acetone (≥ 99.7%, Carl Roth GmbH, Karlsruhe, Germany) before mixing them with 39 mL of distilled water, thereby gaining a stock solution of 1 mM. Dilution series' were done to obtain the final concentrations for imidacloprid of 0.06, 0.6, 1.2 and 2.4 µM which are equivalent to dosages per 10 µL of 0.15, 1.5, 3 and 6ng. For clothianidin, those final concentrations were 0.02, 0.1, 0.2, 0.3, 0.4 and 0.8µM. These are equivalent to dosages per 10 µL of 0.05, 0.25, 0.5, 0.75, 1 and 2ng (doses were chosen according to the results of the toxicity tests, see Chapter V. 4.1.). Controls were fed with 2 M sucrose solution containing the equivalent of acetone of the highest administered dose. The percentage of acetone in the final solution did not exceed 0.01% (v/v). Stock solutions for every concentration were made once at the beginning of the bee season. Of each stock solution batches of one milliliter were extracted and filled in 1.5 mL Rotilabo® micro centrifuge tubes (Carl Roth GmbH, Karlsruhe, Germany) and deep frozen at -18°C. The number of tubes depended on the number of experiments conducted and the concentrations tested in one experiment. Three dosages of Perizin® containing 1, 2 and 5 µg of the active ingredient coumaphos were tested. Solutions were prepared on the day of the experiment diluting 25 / 50 / 125 µL of Perizin® in 8 ml of 33% honey solution (2008) or in 33 % sucrose solution (2009).

To analyze sub-lethal influences of insecticides on individual foragers, the same feeding technique applied in the cage experiments (Chapter VIII.3.2.) was used. In every experiment, the previously tagged bees were caught, after being identified with the USB-Reader-Pen (iID® PEN mini USB, microsensys, Erfurt, Germany), at the feeder site directly after landing. In order to assign the bees to the different dosage groups, the bees were caught and allocated to the experimental groups as follows: The first bee caught was put into group 1 (e.g. control), the second bee into group 2

(dosage x), the third bee into group 3 (dosage y), and so on. This pattern was repeated until all tagged bees were caught and assigned to one of the experimental groups. The maximum number of bees per dosage group was 12. The bees were kept isolated in the tubes for 20 min to avoid trophallaxis with other bees and to observe the possibility of regurgitation. After the treatment, the bees were released at the feeder site.

VIII. 3.6. Data analysis

For statistical analysis SPSS Statistics 17.0 (SPSS Inc., Chicago, Illinois, USA) was used. The analysis of the collected data was done with different self-programmed algorithms (Syntax-files) which will be described in the following (all files mentioned in the following are attached on the Supplementary data disc, and described in Supplemental Material). Before mounting the transponders on the bees, all transponder-IDs to be used were read-out with the help of the USB-Reader-Pen and saved in a text-file. When the bees were caught at the feeder site for insecticide administration, their chip-IDs were assigned to one of treatment doses which were coded by single digit numbers (control= 1, dosage x= 2, dosage y= 3, etc.). The information about the chip-ID and the assigned administration were saved into an SPSS-syntax file (**Fig. 27**).

```
6   RECODE
7    Number
8   ( 10980 = 2 ) ( 10982 = 1 ) ( 10990 = 2 ) ( 10998 = 2 ) ( 11006 = 3 ) ( 11014 = 1 ) ( 11030 = 1 )
9   ( 11044 = 5 ) ( 11046 = 2 ) ( 11054 = 5 ) ( 11078 = 0 ) ( 11086 = 2 ) ( 11091 = 3 ) ( 11094 = 3 )
10  ( 11102 = 1 ) ( 27234 = 1 ) ( 27235 = 1 ) ( 27242 = 0 ) ( 27250 = 1 ) ( 27253 = 0 ) ( 27261 = 5 )
11  ( 27263 = 3 ) ( 27266 = 0 ) ( 27269 = 4 ) ( 27277 = 4 ) ( 27287 = 5 ) ( 27293 = 0 ) ( 27295 = 3 )
12  ( 27298 = 5 ) ( 27317 = 5 ) ( 27325 = 0 ) ( 27327 = 2 ) ( 27333 = 0 ) ( 27346 = 2 ) ( 27359 = 2 )
13  ( 94472 = 0 ) ( 94476 = 5 ) ( 94492 = 4 ) ( 94504 = 3 ) ( 94516 = 4 ) ( 94524 = 1 ) ( 94532 = 5 )
14  ( 94536 = 2 ) ( 94548 = 4 ) ( 94556 = 4 ) ( 94564 = 2 ) ( 94580 = 1 ) ( 94588 = 3 ) ( 94597 = 4 )
15  ( 94617 = 3 ) ( 94623 = 3 ) ( 94626 = 4 ) ( 94639 = 2 ) ( 94661 = 3 ) ( 94665 = 4 ) ( 94671 = 3 )
16  ( 94673 = 1 ) ( 94674 = 5 ) ( 94679 = 4 ) ( 94681 = 3 ) ( 94687 = 0 ) ( 94690 = 0 ) ( 94695 = 5 )
17  ( 94697 = 4 ) ( 94703 = 1 ) ( 94705 = 4 ) ( 94711 = 5 ) ( 94713 = 0 ) ( 94719 = 2 )
18  INTO  Behandlung .
19  EXECUTE.
```

Figure 27: Screenshot of a Recoding-Syntax. This Syntax creates a new variable which recodes the TagID-Number (represented by the last five digits) into its assigned treatment. In this case: 0= Bees that were tagged but did not return to the feeder on the day of treatment, 1= control, 2= Imidacloprid 0.15ng, 3= Imida 1.5ng, 4= Imida 3ng, 5= Imida 6ng.

Subsequent to every observation-period the reader data were read out with software supplied by Microsensys GmbH, Erfurt, Germany, and saved on a notebook as a

text-file. The next step was to import this file into a SPSS datasheet with a predefined ".tpf" (TextWizard Predefined Format). From here the processing of the data started. A filter algorithm (**Fig. 28**) searched for and erased rapid-succession-readings of the same transponder-ID at antennas of the same reader, which occurred when a tagged bee lingered beneath the scanners for too long e.g. being blocked by flow of opposing traffic. The calculation of the time durations of the different foraging phases (see also Chapter VIII.3.3.2.2) required an intermediate step. Since registrations

```
11  Sort cases by
12  Number (d) date (d) time (d).
13  USE ALL.
14  COMPUTE filter_$=(tag<>lag(tag) | antenna<>lag(antenna)  | Scanner_ID<>lag(Scanner_ID) | time<lag(time)-2).
15  VARIABLE LABEL filter_$ 'tag<>lag(tag) | antenna<>lag(antenna)  | Scanner_ID<>lag(Scanner_ID) | time<lag(time)-2 (FILTER)'.
16  VALUE LABELS filter_$  0 'Nicht ausgewählt' 1 'Ausgewählt'.
17  FORMAT filter_$ (f1.0).
18  FILTER BY filter_$.
19  EXECUTE.
20  FILTER OFF.
21  USE ALL.
22  SELECT IF(filter_$).
23  EXECUTE.
```

Figure 28: **Screenshot of the Filter Syntax.** After sorting the variables Number (Chip-ID), date, and time in a descending order the syntax erases all unnecessary antenna registrations of the same Chip-ID at the same Scanner in a time frame of two seconds after the first registration.

saved on the scanner are presented in a descending order with the last registration at the top, they are displayed in the same fashion after the import to SPSS. Successive registrations along both antennas of the same scanner can happen in the course of a second, giving both registrations exactly the same time-stamp. While the data is ordered in a descending way, these registrations remain in the correct order. However, for the calculations of the time durations it was necessary to switch the order between descending and ascending, which leads to a disorder of the registrations with the same time-stamp. Therefore, a new variable was introduced which numbered the cases consecutively after they were sorted in a descending fashion by Tag-Number, Date, and Time. Thus, changing the order of this variable avoided a disorder of the data. The total duration of a complete foraging trip and the time interval spent inside the hive between foraging trips was calculated from hive-data, the duration for a stay inside the feeder compartment was calculated from the feeder-compartment- scanner-data, and the fight time from hive to feeder and back again was calculated from the combination of both. Three different algorithms were required: one for calculation of foraging trip duration and flight times back and forth, one for feeder stay duration, and one for in-hive intervals (for more information on the syntax files that were used see also Supporting Information, Syntax files). Non-

parametrical Kruskal-Wallis and Mann-Whitney-U-tests were used to compare the foraging activity at the feeder, the median total time needed for a foraging flight, the flight time to the feeder, the flight time to the hive, the time spent at the feeder, and the interval between foraging flights for every bee in the different treatement groups. The null hypothesis was rejected at the 5% -level ($p < 0.05$).

VIII. 4. Results

VIII. 4.1. Toxicity Tests

Immediately after administration of the highest dose of 6ng, symptoms of poisoning were observable. These bees were running around the cage with an awkwardly arched abdomen, a symptom also observed for bees treated with 3ng, and to some extend for the 1.5ng group at the 2h observation time. A second symptom, in addition to the arched abdomen, was observable especially after treatment with 3ng and 6ng. Bees were lying on their backs while moving their legs in what seemed to be a running motion.

The abdomens of the dead bees collected from every 6ng-cage had turned black and the bees seemed to be drenched in an indiscernible liquid (**Fig. 29 A**). This phenomenon was never observed for dead bees form the other test-cages (**Fig. 29 B**) The LD_{50}(24h) over all experiments combined was 2.648 ng/bee (StD: +/- 0.525) with a range from 2.244-3.41 ng/bee. The natural response rate (probability of observing a response, in this case death, with no dose applied) was 3.3% +/- 1.8 SEM. The LD_{50}(48h) over all experiments was found to be somewhat lower at 2.528ng/bee (StD: +/-0.5245) with a range from 1.999 - 3.048ng/bee. The natural response rate was 21% +/-4.7 SEM. The high natural response rate after 48h can be considered a result of testing bees of unknown age, since the mortality rate increased throughout all groups (see also **Table S 1**, Supporting Information).

Considering these results, the range of tested doses was chosen between 0.05 – 2ng/bee. The low threshold is thought to be a dose to which bees might be exposed through nectar and pollen, while the high threshold was chosen close to the lowest LD_{50} value found in these cage tests after 48h.

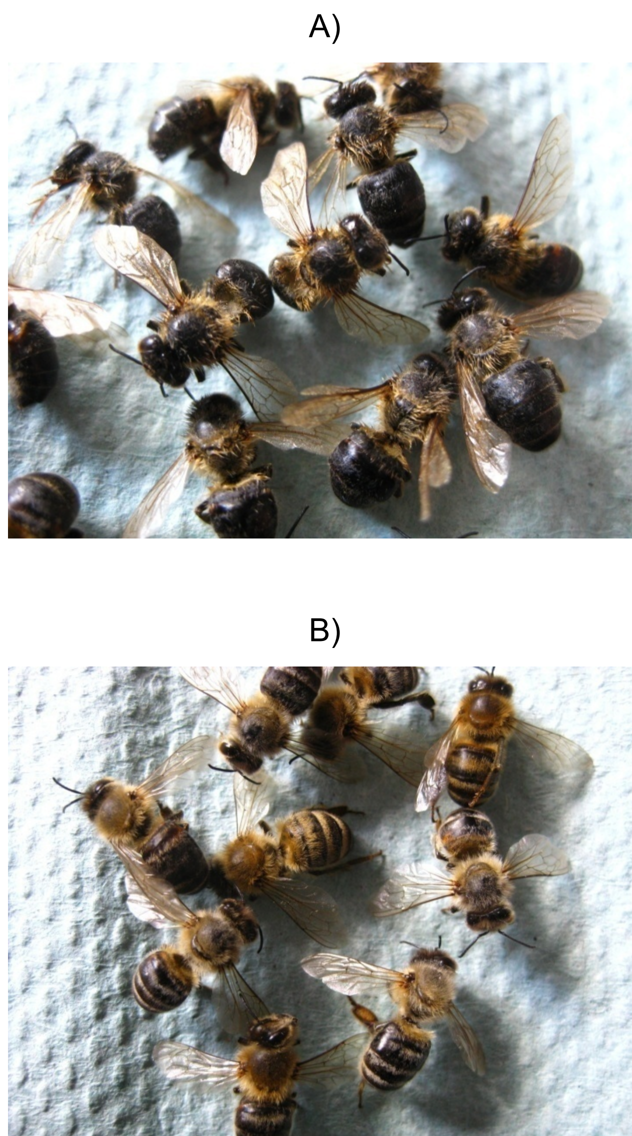

Figure 29 Samples of dead bees collected from the cages. (A) Drenched bees with blackened abdomen collected from a cage containing bees treated with 6ng in comparison to (B) bees collected from a cage containing bees treated with 3ng.

VIII. 4.2. Foraging Experiments

Due to the knowledge of sublethal effects of imidacloprid in foraging behavior (Bortolotti et al. 2003; Yang et al. 2008) the number of trials was reduced to one each year (**Tab. 9)** in order to calibrate and validate this method, and to have more time for trials focusing on the effects of clothianidin and coumaphos, substance not yet known for potential sub-lethal properties.

Table 9: List of the trials conducted with different substances, monitoring method, and dosage groups over 3 years.

Date	Substance	Dosage groups	N Bees	Monitoring at feeder
15.07.2008	Imidacloprid	C; 3ng	10; 9	USB-Pen
21.07.2008	Coumaphos	C; 5µg	9; 9	USB-Pen
28.07.2008	Coumaphos	C; 5µg	10; 10	USB-Pen
05.08.2008	Coumaphos	C; 2µg	10; 10	USB-Pen
11.08.2008	Coumaphos	C; 2µg	10; 10	USB-Pen
18.08.2008	Coumaphos	C; 1µg	10; 10	USB-Pen
25.08.2008	Coumaphos	C; 1µg	10; 10	USB-Pen
08.07.2009	Clothianidin	C; 1ng; 2ng	11; 11; 12	Feeder compartment
14.07.2009	Clothianidin	C; 0.5ng; 1ng	9; 10; 10	Feeder compartment
20.07.2009	Clothianidin	C; 0.05ng; 0.5ng; 1ng	11; 10; 10; 11	Feeder compartment
03.08.2009	Clothianidin	C; 0.05ng; 0.5ng; 1ng	10; 12; 12; 11	Feeder compartment
10.08.2009	Coumaphos	C; 1µg; 2µg; 5µg	13; 13; 13; 13	Feeder compartment
17.08.2009	Coumaphos	C; 1µg; 2µg; 5µg	13; 13; 13; 13	Feeder compartment
24.08.2009	Clothianidin	C; 0.05ng; 0.5ng; 1ng	12; 12; 11; 11	Feeder compartment
01.09.2009	Imidacloprid	C, 0.15ng, 1.5ng, 3ng, 6ng	11; 12; 12; 12; 12	Feeder compartment
01.07.2010	Imidacloprid	C, 1.5ng	7; 7	Feeder compartment
07.07.2010	Clothianidin	C; 0.05ng; 0.5ng; 1ng	8; 8; 8	Feeder compartment
15.07.2010	Clothianidin	C; 0.05ng; 0.5ng; 1ng; 2ng	10; 9; 9; 10; 10	Feeder compartment
20.07.2010	Clothianidin	C; 0.05ng; 0.5ng; 1ng; 2ng	11; 11; 12; 12; 12	Feeder compartment
26.08.2010	Clothianidin	C; 0.05ng, 0.25ng; 0.5ng; 0.75ng	10; 10; 8; 9; 8	Feeder compartment

VIII. 4.2.1 Return rates to the hive after treatment

In the trials with imidacloprid conducted from 2008-2010 (**Tab.9**), all or almost all bees returned to the hive after post-treatment release at the feeder in the control

groups and in the groups treated with doses up to 3ng. However, only a quarter of the bees treated with 6ng returned (controls, 0.15 and 1.5ng: 100%; 3ng: 95%, 6ng 25%). Of the bees not registered at the hive or the feeder within the three-hour observation period after release, none was registered during the following days. Among the bees treated with 3ng and 6ng that were not directly flying to the hive, we observed reduced motor activity, followed by a phase of immobility with occasional trembling movements around the feeder area.

Similar to trials with imidacloprid, in trials with clothianidin conducted during 2009, most of the bees returned to the hive after treatment with dosages up to 0.5ng (controls and 0.05ng 100%; 0.5ng 95%). At a higher dose of 1ng, only 70% of the bees returned to the hive during the following three-hour observation period, and only 25% returned after the uptake of 2ng. The bees that did not return within this period were neither registered again at the hive nor at the feeder during the following days (**Tab. 10**). We observed strong abnormal reactions to the highest dose of 2ng after release at the feeder, characterized by an awkwardly arched abdomen, followed by turning upside down and lying on the back with paddling leg movements.

Table 10: Number of bees that returned to the hive/Number of bees that were treated over all experimental trials conducted with Clothianidin. (x= dose not used in this experiment)

	2009					2010			Total
	Exp1	Exp2	Exp3	Exp4	Exp5	Exp6	Exp7	Exp8	
Control	11/11	9/9	11/11	10/10	12/12	9/9	10/10	11/11	83/83
0.05ng	x	x	10/10	12/12	11/11	8/8	9/9	11/11	61/61
0.5ng	x	7/10	10/10	12/12	11/11	7/8	9/9	12/12	68/72
1ng	8/11	5/10	8/11	8/11	7/11	7/8	8/10	11/12	62/84
2ng	4/12	x	x	x	x	x	3/10	2/12	7/34

In the trials conducted to test the influence of coumaphos, a total number of 221 bees, divided into 84 controls, 46 1µg-bees, 46 2µg-bees, and 45 5µg-bees, were treated and released into the registration colonies. Over the course of 48 hours, all of the control- and 1µg-bees (100%), 97.8% of the 2µg-, and 93.3% of the 5µg-bees were registered again at the hive entrance. Over two years we conducted a total of eight experiments during which 26634 foraging trips (2008: 13584, 2009: 13050) were registered. In **Table 11**, the number and proportion of treated bees that returned to the feeder after administration in the different experiments is shown.

Tabelle 11: Number and proportion (in %) of treated bees returning to the feeder in the different experiments after administration. "Distance" marks the range between hive and feeder.

	Imida 2008: distance 7m			**Imida 2009: 7m**			**Imida 2010: 7m**		
Dose	N (%): a.t.	24h a.t.	48h a.t.	a.t.	24h a.t.	48h a.t.	a.t.	24 h a.t.	48 h a.t.
Control	10 (100)	9 (90)	9 (90)	11 (100)	12 (100)	12 (100)	7 (100)	7 (100)	6 (86)
0.15ng	-	-	-	12 (100)	11 (92)	11 (92)	-	-	-
1.5ng	-	-	-	12 (100)	12 (100)	12 (100)	7 (100)	7 (100)	6 (86)
3ng	9 (100)	9 (90)	9 (90)	7 (58)	10 (83)	10 (83)	-	-	-
6ng	-	-	-	0 (0)	3 (18)	3 (18)	-	-	-

	Clothia 2009: distance 7m		**Clothia 2010: 7m**	
Dose	N (%): a.t.	24h a.t.	a.t.	24h a.t.
Control	53 (100)	47 (89)	30 (100)	26 (87)
0.05ng	33 (100)	30 (91)	28 (100)	26 (93)
0.5ng	36 (85)	31 (73)	28 (100)	25 (89)
1ng	31 (61)	27 (59)	24 (83)	26 (90)
2ng	0 (0)	0 (0)	4 (18)	4 (18)

Clothia 2010: 370m	
Dose	a.t.
C	10 (100)
0.05ng	10 (100)
0.25ng	8 (100)
0.5ng	9 (100)
0.75ng	4 (50)

	Couma 2008: distance 7m				**Couma 2009: 7m**		
Dose	N (%): a.t.	24h a.t.	48h a.t.	Dose	a.t.	24h a.t.	48h a.t.
Control (1µg)	20 (100)	20 (100)	17 (85)	**C**	26 (100)	25 (96)	23 (87)
Control (2µg)	20 (100)	20 (100)	19 (95)	**1µg**	28 (100)	26 (100)	24 (92)
Control (5µg)	18 (100)	17 (94)	17 (94)	**2µg**	25 (96)	25 (96)	24 (92)
1µg	20 (100)	19 (95)	16 (80)	**5µg**	21 (84)	22 (88)	20 (80)
2µg	19 (100)	19 (100)	17 (89)				
5µg	15 (79)	18 (95)	16 (84)				

The measured median time for a complete foraging trip made by untreated bees was 92.5 seconds in 2008 without the use of the feeder compartment, and 109 seconds in 2009 when the feeder compartment was introduced. With the reading devices at the entrance of the feeder compartment it took the bees a median time of nine seconds from leaving the hive until entering the feeder compartment. The a median duration of a stay at the feeder was 81.75 seconds, and the median time from leaving the feeder compartment until re-entering the hive was 10 seconds.

VIII. 4.2.2 Number of feeder visits during observation periods

Once a bee was consecutively registered at hive entrance and at the feeder compartment during the three-hour observation periods, it was marked as a feeder visit. The number of feeder visits was determined for every bee at the end of the

observation periods, and will be defined as foraging activity in the following. All increases and decreases listed below relate to the comparison of the median number of feeder visits.

Imidacloprid (foraging activity):

Administration of 1.5ng and 3ng Imidacloprid significantly reduced foraging activity immediately after treatment in every experiment conducted (**Fig. 30, Imidacloprid**). After 24 hours, this effect was not found to be persistent. When the foraging activity was monitored with the USB-Pen in 2008, the number of feeder visits for bees treated with 3ng imidacloprid was reduced by 22% ($p_{3nga.t.} \leq 0.01$, Mann-Whitney-U-test). In the first experiments with the feeder compartment in 2009 administration of 3ng reduced the number of feedervisits by 98% ($p_{3ng} \leq 0.001$, Mann-Whitney-U-test) compared to the control. Administration of 1.5ng led to a reduced foraging activity by 43% in the experiments in 2009 ($p_{1.5nga.t.} \leq 0.001$, Mann-Whitney-U-test) and by 67% in 2010 ($p_{1.5nga.t.} \leq 0.001$, Mann-Whitney-U-test). Not all bees treated with 3ng reappeared at the feeder immediately after treatment. However, all but two returned regularly after 24h. Bees treated with 6ng, which had reentered the hive after release, did not visit the feeder until 24h after treatment.

Clothianidin (foraging activity):

Administration of 0.5ng, 0.75ng, 1ng, and 2ng clothianidin resulted in a significant reduction in the number of feeder visits compared to the control group during the three-hour observation period immediately after treatment (**Fig. 30, Clothianidin**). Twenty-four hours after administration, no statistically verifiable effects on the foraging activity were detected in any of the treatment groups. During the trial series in 2009, administration of 0.5ng and 1ng reduced number of feeder visits by 22% ($p_{0.5nga.t.} \leq 0.001$, Mann-Whitney-U-test) and 60% ($p_{1nga.t.} \leq 0.001$, Mann-Whitney-U-test), respectively. The repetitive short-distance experiments conducted during the following year showed that 1ng and 2ng clothianidin reduced the number of feedervisits per bee by 79% ($p_{1nga.t.} \leq 0.001$, Mann-Whitney-U-test) and 67% ($p_{2nga.t.} \leq 0.001$, Mann-Whitney-U-test), respectively. The administration of 0.5ng in these short-distance tests reduced the visit frequency by 18% ($p_{0.5nga.t.} = 0.028$, Mann-Whitney-U-test). When applied in the long distance experiment, 0.5ng led to a

significant reduction of the foraging activity by 59% ($p_{0.5nga.t.} \leq 0.001$, Mann-Whitney-U-test).

Only about 60% of the bees treated with 1ng (≈1/4 LD_{50}) in 2009 returned to forage from the feeder (see also **Tab. 11**). The missing bees were neither registered again at the hive nor at the feeder over the following days. In 2010 about 83% of the bees treated with 1ng returned to the feeder immediately after administration and the proportion was increased to 90% on the day after treatment. Bees treated with 2ng clothianidin that returned to the hive after release did not visit the feeder and did not register at the hive entrance again. The lowest dose used for clothianidin (0.05ng), had no detrimental effects on the mean number of feeder visits.

Coumaphos (foraging activity):

Coumaphos led to a reduction of the visit frequency of bees treated with 5µg and 2µg/bee (**Fig. 30, Coumaphos**). The reduction in the number of feeder visits was still detectable after 24 hours and even after 48 hours for 5µg. During the experiments with the USB-Pen in 2008, the number of visits was reduced by 87% and 23% respectively ($p_{5µga.t.} \leq 0.001$; $p_{2µga.t.} \leq 0.01$; Mann-Whitney-U-test) immediately after administration of 5µg and 2µg. Observations during the observation-period on the following day revealed the persistence of the effect of 5µg and 2µg coumaphos on the visit frequency. The number of visits was still reduced by 89% for the 5µg group ($p_{5µg24a.t.} \leq 0.001$, Mann-Whitney-U-test) and by 23% for 2µg group ($p_{2µg24a.t.} \leq 0.001$, Mann-Whitney-U-test) after 24 hours. Furthermore, at the end of the 48 hour observation period a reduction of the visit frequency by 73% ($p_{5µg48a.t.} \leq 0.001$, Mann-Whitney-U-test) was still persistent for the bees treated with 5µg coumaphos, but no difference was observed between the 2µg group and control ($p = 0.169$, Mann-Whitney-test).

In 2009, when the feedercompartment was introduced the results were similar to the year before except for the 48h observation period. Both 5µg and 2µg coumaphos significantly reduced the visit frequency at the feeder immediately after (5µg: -82%, $p_{5µga.t.} \leq 0.001$; 2µg: -13%,$p_{2µga.t.} \leq 0.01$, , Mann-Whitney-U-test) and 24h after treatment (5µg: -15%, $p_{5µg24a.t.} \leq 0.001$; 2µg: -15%, $p_{2µg24a.t.} \leq 0.001$, Mann-Whitney-U-test). After 48h, no significant effects were observed for either dose ($p_{5µg48a.t.}=0.306$; $p_{2µg48a.t.}=0.079$, , Mann-Whitney-U-test). In both years, administration of 1µg/bee had no adverse effect on foraging activity.

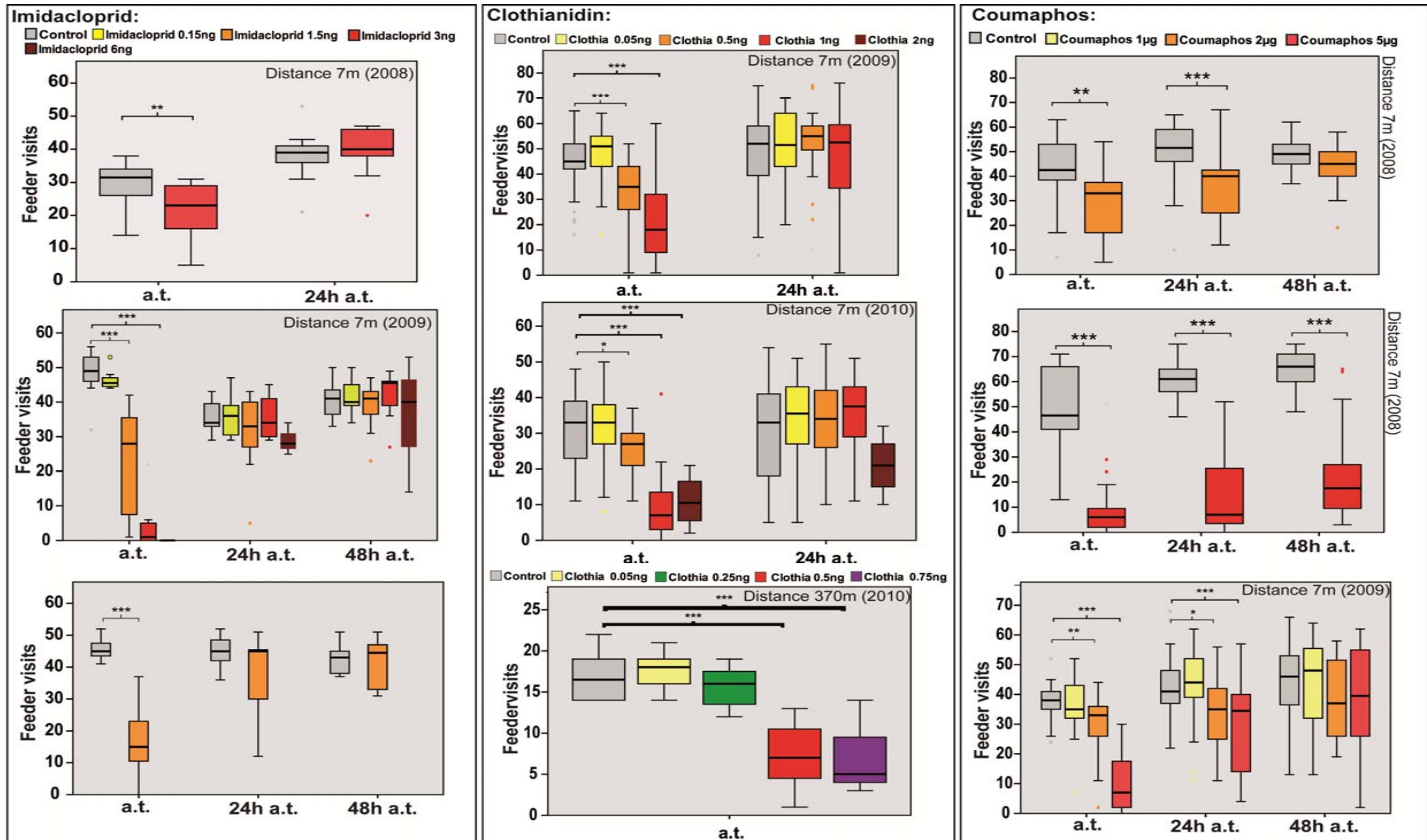

Figure 30 Influence on the number of feeder visits after treatment with imidacloprid, clothianidin, and coumaphos. Ordinate: Number of feeder visits per bee. Abscissa: different observation periods. a.t.= immideately after treatment, 24h a.t.= 24h after treatment, 48h a.t.= 48h after treatment. During 3-hour observation periods, the frequency of visitation at the feeder was recorded. Oral administration of the lowest dose of each substance had no influence on the number of feeder visits per bee. Treatment with 1.5 and 3ng imidacloprid, 0.5ng and 1ng clothianidin, or 2µg and 5µg coumaphos led to a significant reduction of the foraging activity. For the neonicotinoid substances the observed effect usually did not persist over the following days. Nevertheless, the influence of coumaphos was still detectable ≥ 24 hours after administration. (*= $p < 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$, Mann-Whitney-U-test).

VIII. 4.2.3 Analysis of multiple parameters/phases of foraging

VIII. 4.2.3.1 Total duration of foraging trips

The median total duration for a single foraging trip was described as the period that a tagged bee needed from leaving the hive until returning to it. This parameter was measured during the experiments with the USB-Pen and the feedercompartment respectively.

Imidacloprid (Total foraging duration):

During three experiments conducted from 2008 until 2010, the administration of 1.5ng and 3ng imidacloprid, respectively, significantly prolonged the median duration needed for a single foraging trip during the initial three-hour observation period immediately after treatment but did not persist for 24 hours (**Fig. 31 A**). While using the USB-Pen-Method the influence of 3ng was tested against a control group. It took the imidacloprid-treated bees 50% longer to complete a foraging trip ($p< 0.01$, Mann-Whitney-U-Test). The feedercompartment-method revealed that bees treated with 1.5ng and 3ng imidacloprid needed 50% and 130% longer, respectively for a single foraging trip ($p_{1.5ng}\leq 0.001$; $p_{3ng}< 0.001$, Kruskal-Wallis-Test followed by Mann-Whitney-U-Test). The highest dose of 6ng led to an almost complete ceasing of foraging activity. Only one bee was registered again at the hive entrance after returning, but did not make it to the feeder. In a concluding experiment in 2010, imidacloprid at a dose of 1.5ng lead to an extended foraging trip immediately after administration (+30%, $p\leq 0.01$, Mann-Whitney-U-Test).

Clothianidin (Total foraging duration):

Similar to the influence of 1.5ng and 3ng imidacloprid, the results received from the nine experiments, during 2009 and 2010, conducted with clothianidin showed that administration of 0.5ng and 1ng had a prolonging effect on the median duration of a single foraging trip immediately after administration (**Fig. 31 B**). Bees treated with these doses in 2009 needed 10% and 23% longer for a foraging trip compared to the control bees ($p_{0,5ng}< 0,05$; $p_{1ng}\leq 0,001$, Kruskal-Wallis-Test followed by Mann-Whitney-U-Test). In the following year the same doses induced longer foraging trips by 15% ($p< 0.05$) and 33% times ($p\leq 0.001$). Immediately after treatment with the lowest dose of clothianidin (0.05ng), no significant effects on the total duration of a

foraging trip were induced in both experimental years. However, in 2009 it was observed that the control-bees needed longer than the 0.05µg- (+10%, $p \leq 0.01$) and 0.5µg-bees (+10%, $p \leq 0.01$) 24 hours after administration. This effect did not show in the experiments conducted a year later. Extending the feeder-hive distance from originally seven to 370 meters reinforced the effect of 0.5ng clothianidin by prolonging a foraging trip of a treated bee by 45% ($p \leq 0.001$). The administration of 0.75ng extended the median duration of a foraging trip by 37%. Due to the reduced N number after treatment (n=4) this result was not statistically reliable ($p = 0.188$).

Coumaphos (Total foraging duration):

A detrimental influence of coumaphos on the median foraging trip duration was also found for the highest administered dose of 5µg (**Fig. 31 C**). Both experiments conducted with the USB-Pen-method revealed that bees treated with this dose needed 20% more time ($p < 0.05$, Mann-Whitney-U-Test) for a complete foraging trip during the three-hour observation period immediately after treatment. This effect was still persistent 24- and 48 hours after administration with the median duration of a foraging trip being prolonged by 17% ($p < 0.05$) and 25% ($p \leq 0.001$) respectively. Administration of 1µg and 2µg coumaphos did not affect the foraging trip duration significantly (**Fig. S2**, Supplementary Material). The experiments conducted with the feeder compartment-method the following year, revealed prolonged foraging trip duration by 12% ($p \leq 0.01$) during the observation period immediately after treatment. In contrast to results from the previous year this effect was neither persistent after 24- nor 48 hours. Twenty-four-hours after the feeding with 1µg, we observed a trend for shorter foraging trip durations (-11%) in comparison to the control which was, although narrowly, not statistically supported ($p = 0.051$).

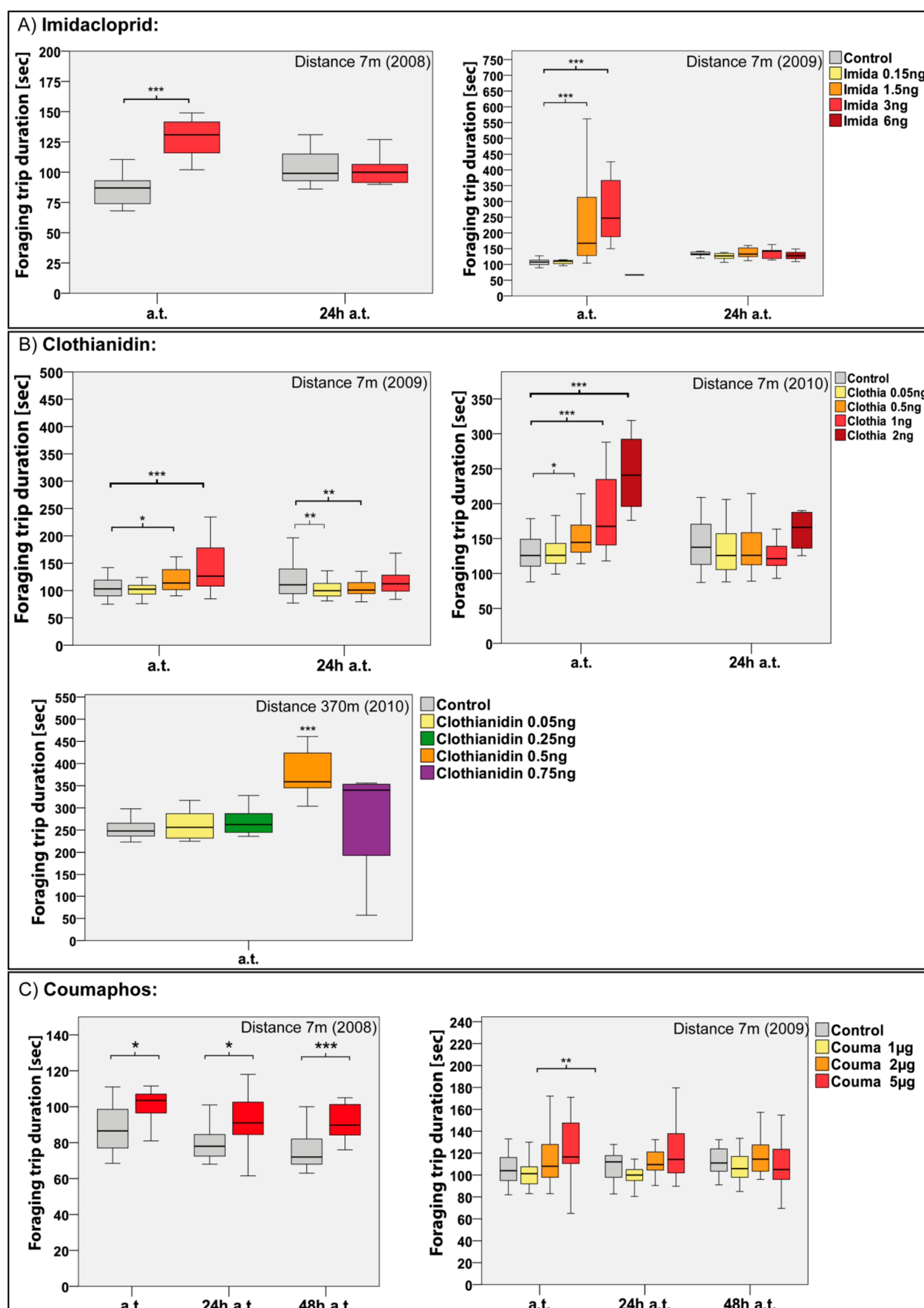

Figure 31: **Duration for a foraging trip after treatment with imidacloprid (A), clothianidin (B), and coumaphos (C).** Ordinate: Median time in seconds. Abscissa: different observation periods. a.t.= immediately after treatment, 24h a.t.= 24h after treatment, 48h a.t.= 48h after treatment. Plotted was the median duration for a total foraging trip made by every bee during 3-hours observation periods. *= p< 0.05, **= p≤ 0.01, ***= p≤ 0.001. (**A**) Bees treated with 1.5ng and 3ng imidacloprid spent more time outside the hive during the first three hours immediately after treatment. This effect was not persistent after 24h regardless of the year. (**B**) After bees were treated with 0.5ng, 1ng, and 2ng clothianidin their median time spent outside the hive was significantly prolonged compared to the control group. After 24h in 2009, statistically significant shorter foraging trip durations were observed by the bees treated with 0.05ng and 0.5ng. (**C**)Foraging trips by the bees treated with 5µg coumaphos in 2008 were found to be significantly prolonged even 48h post administration. In 2009 the prolonging effect was only observable during the 3-hour observation period immediately after treatment with 5µg and not persistent after 24h.

VIII. 4.2.3.2 Flight duration from hive to feeder

With the introduction of the feeder compartment it was possible to determine the time a bee needed from leaving the hive until arriving at the feeder and vice versa. The time interval between registration at the hive and subsequent registration at the feeder compartment phase will be referred to in the following as flight duration to the feeder.

Imidacloprid (Flight duration Hive ► Feeder):

Bees treated with 1.5ng and 3ng Imidacloprid needed extended time to get from the hive entrance to the feeder compartment during the observation period immediately after administration (**Fig. 32 A**). Either after 24- or 48 hours no effect was detected compared to the controls. In 2009 treatment with 1.5ng or 3ng imidacloprid prolonged a flight to the feeder by 64.7% and 241.1%, respectively ($p_{1.5ng} \leq 0.01$; $p_{3ng} \leq 0.001$, Kruskal-Wallis-Test followed by Mann-Whitney-U-Test). The effect on bees treated with 1.5ng was even more profound in 2010, when administration extended the flight time by 125% ($p \leq 0.01$). This effect was found to be persistent, to a lesser extent, after 24h in 2009 for the 1.5ng-bees ($p < 0.05$) but not in the following year ($p = 0.71$).

Clothianidin (Flight duration Hive ► Feeder):

The short-distance (Feederdistance 7 meters) experiments with clothianidin did not reveal any detrimental effect on this phase of a foraging trip. Rather, bees treated with the lowest dose of 0.05ng tended to get to the feeder compartment faster. However the long distance trial prolonged the flight duration for bees treated with 0.5ng and 0.75ng and did not give any evidence that 0.05ng reduces this phase (**Fig. 32 B**).

Administration of 0.05ng reduced median flight time by 28.2% compared to the controls ($p < 0.05$) in the 2009 short-distance experiments. Nevertheless, this possible stimulation effect was neither observed during the short- (-4.8%, $p = 0.784$) nor the long-distance experiments (+3.6%, $p = 0.739$) conducted as a repetition the following year. When the feeder was 370 meters away from the hive, the bees treated with 0.5ng and 0.75ng took 17% and 50% ($p_{0.5ng} \leq 0.01$; $p_{0.75ng} \leq 0.01$) longer, respectively to fly to the feeder.

Coumaphos (Flight duration Hive ► Feeder):

Even though there was some variation observable immediately after administration of 5µg coumpahos in 2009, as well as a tendency for reduced median flight durations for bees treated with 2µg, no statistically verifiable differences concerning flight duration to the feeder compartment were found after treatment with coumaphos, regardless of the dose administered (**Fig. 32 C**).

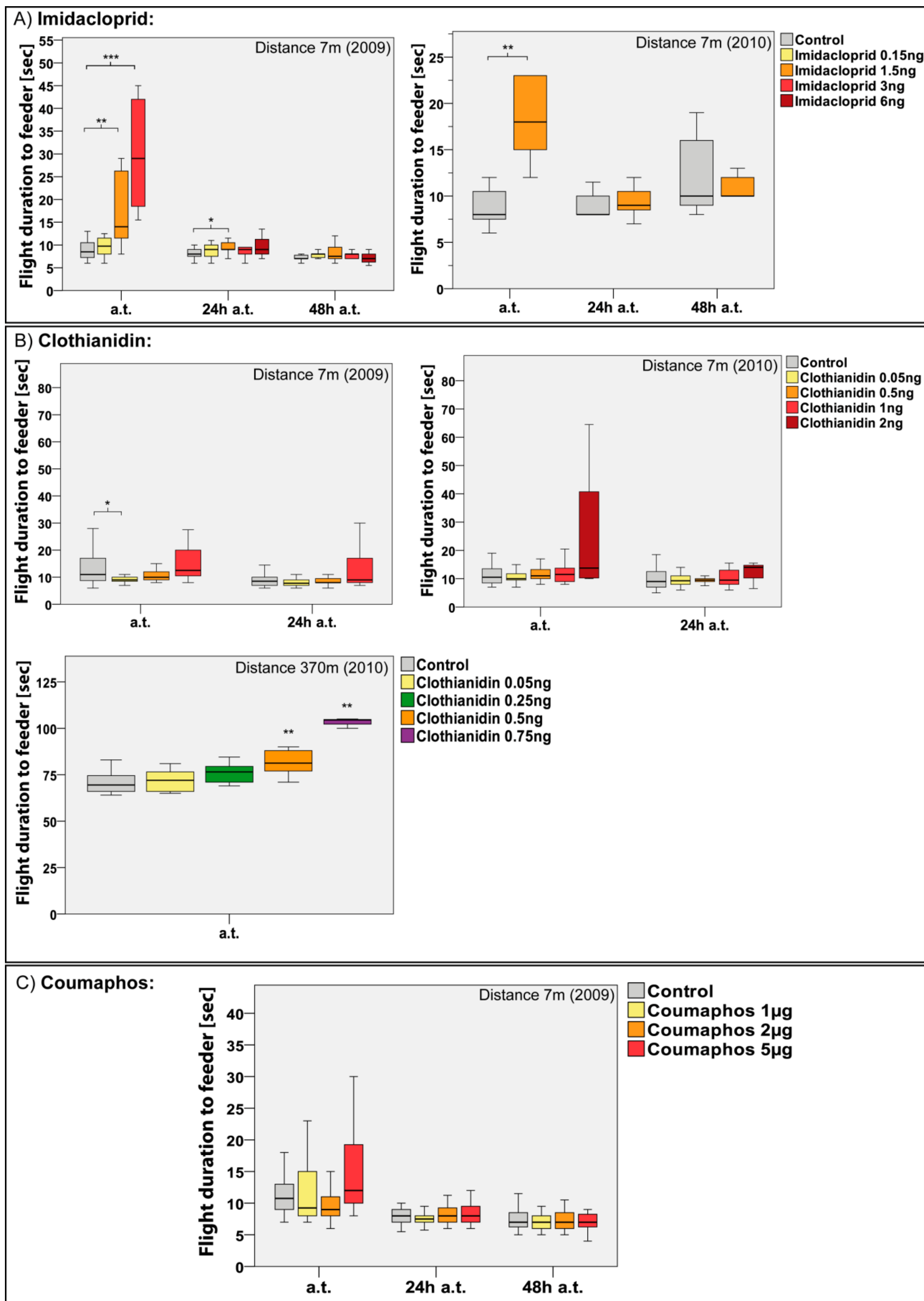

Figure 32: Effect of imidacloprid (A), clothianidin (B), and coumaphos (C) on the flight duration to the feeder. Plotted is the median time in seconds that each bee needed in order to fly from the hive to the feeder during 3-hours observation periods immediately after treatment (a.t.), 24h (24h a.t.) after treatment, and up to 48h (48h a.t.) after treatment. *= $p < 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$.

(A) A significantly prolonged flight duration to the feeder was observed immediately after oral administration of 1.5ng and 3ng imidacloprid compared to the control group. (B) Treatment with clothianidin, regardless of the dose, showed no significant prolonging effect on median flight duration of a bee to the feeder in the short-distance experiments. Nevertheless, in 2008 it took the bees of the 0.05ng group significantly less time to get to the feeder immediately after treatment. Administration of 0.5ng and 0.75ng induced significantly longer flights to the feeder when it was positioned in further away (370 meters). (C) Treatment with coumaphos did not affect the flight duration to the feeder at any time after administration.

VIII. 4.2.3.3 Duration of stay at the feeder

The moment from entering the feeder compartment until leaving it again marked the duration of stay at the feeder by a transponder-marked bee. This was the longest phase during a foraging trip.

Imidacloprid (Duration of stay at ther feeder):
The median duration of a stay inside of the feeder compartment was significantly prolonged immediately after the administration of 1.5ng and 3ng imidacloprid (**Fig. 33 A**). This effect was not persistent after 24 hours, though. Bees that were treated with the abovementioned doses needed 27.5% and 45.6% more time, respectively, before leaving the feeder compartment again ($p_{1.5ng} < 0.05$; $p_{3ng} < 0.05$, Kruskal-Wallis-Test followed by Mann-Whitney-U-Test). This was in accordance with findings in the following year where bees treated with 1.5ng spent 40% more time inside the feeder compartment compared to the control-bees ($p \leq 0.01$).

Clothianidin (Duration of stay at the feeder):
During the experiments in 2009 and 2010 administration of 0.5ng, 0.75ng, 1ng, and 2ng clothianidin were found to prolong the time spent inside the feeder compartment immediately after administration (**Fig. 33 B**). Nevertheless, this effect was not found to be persistent after 24 hours in either year. However, shortened stays in the feeder compartment were found for bees treated with 0.05ng and 0.5ng during the 24h a.t. observation period in 2009. Administration of 1ng clothianidin in 2009 prolonged the median time spent at the feeder per bee by 22% ($p \leq 0.001$) immediately after treatment. Twenty-four hours after oral administration of 0.05ng and 0.5ng, bees spent 14% ($p \leq 0.01$) and 16.5% ($p \leq 0.01$) less time inside the feeder compartment, respectively. Immediately after treatment with 0.5ng, 1ng and 2ng in 2010 the duration of a stay inside the feeder compartment was extended by 17% ($p_{0.5ng} < 0.05$), 39% ($p_{1ng} \leq 0.001$), and 71% ($p_{2ng} \leq 0.001$), respectively. The long distance experiment in 2010 revealed a similar effect by prolonging the stay inside the feeder compartment of bees treated with 0.5ng-and 0.75ng by 55% ($p_{0.5ng} \leq 0.001$) and 45% ($p_{0.75ng} \leq 0.01$), respectively.

Coumaphos (Duration of stay at the feeder):

Immediately after administration of 5µg coumaphos per bee, the median duration inside the feeder compartment was found to be significantly prolonged (**Fig. 33 C**). A stay during this period was prolonged by 14% for bees treated with this dose ($p \leq 0.001$). The effect was not found to be persistent 24 hours after treatment. Administration of the other dosages had no significant effect on the duration of this phase.

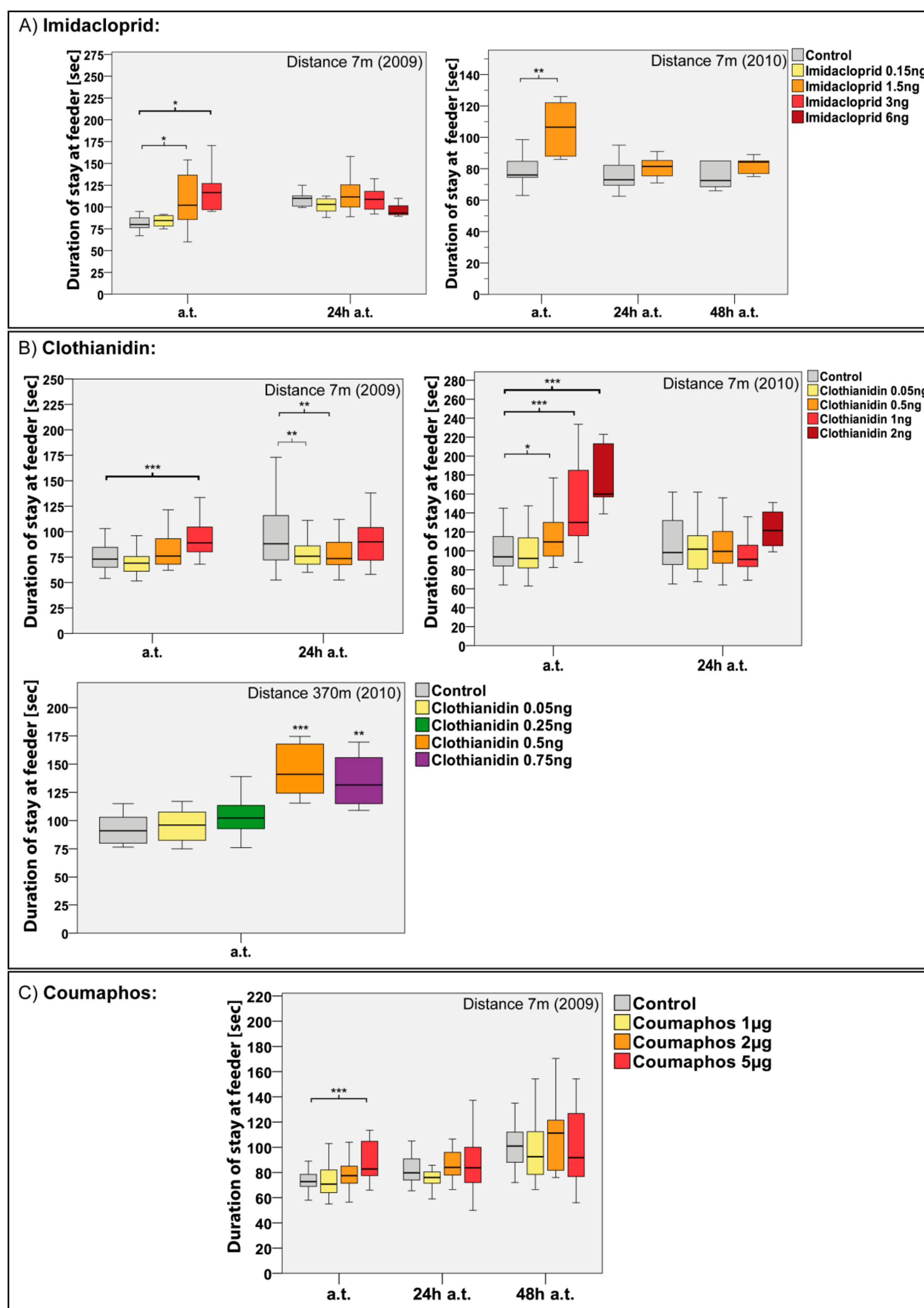

Figure 33: Effect of imidacloprid (A), clothianidin (B), and coumaphos(C) on the duration of stay at feeder. Plotted is the median duration each bee spent inside the feeder compartment in seconds during 3-hours observation periods immediately after treatment (a.t.) and up to 48h after treatment (24h a.t., 48h a.t.). *= p< 0.05, **= p≤ 0.01, ***= p≤ 0.001. (**A**) Bees treated with 1.5ng and 3ng imidacloprid spent more time inside the feeder compartment compared to the control group during the first 3 hours after treatment. This effect was not found to be persistent 24 hours after administration. (**B**) Immediately after treatment with 1ng clothianidin in 2009 and 0.5ng, 1ng, and 2ng clothianidin in 2010, bees spent more time inside the feeder compartment compared to the control group. Twenty-four hours after treatment we observed slightly but significantly shorter feeder visits for bees treated with 0.05ng and 0.5ng when compared to the control, while in 2010 this difference was not found to be persistent after 24h for bees treated with any of these doses. (**C**) Oral administration with 5µg coumaphos resulted in longer stays inside the feeder compartment immediately after treatment, an effect not persistent after 24h.

VIII. 4.2.3.4 Flight duration from the feeder to the hive

The time interval between registration at the feeder and subsequent registration at the hive entrance was defined as flight duration to the hive.

Imidacloprid (Flight duration feeder ► hive):
Similar to the results for the flight duration to the feeder, it was shown that 1.5ng and 3ng significantly extended the flight time back to the hive (**Fig. 34 A**). Again, this adverse effect was not found to be persistent after 24 hours. In 2009 the median time for a homing flight was prolonged by 20% and 210%, respectively ($p_{1.5ng} \leq 0.001$; $p_{3ng} \leq 0.001$, Kruskal-Wallis-Test followed by Mann-Whitney-U-Test). In the following year, administration of 1.5ng led to an extension of the homing flight by 39% ($p \leq 0.001$).

Clothianidin (Flight duration feeder ► hive):
In contrast to the results showing that clothanidin had no adverse effect on the flight time to the feeder at short distances, it was observed that a dose of ≥0.5ng extended the duration of the homing flight significantly immediately after oral administration, an effect not found to be persistent 24 hours later (**Fig. 34 B**). In 2009, the median flight time back to the hive for bees treated with 0.5ng and 1ng was prolonged by 33% for either dose ($p_{0.5ng} \leq 0.001$; $p_{1ng} \leq 0.01$), while in 2010 treatment with 0.5ng, 1ng, and 2ng prolonged the median duration of homing flights by 23%, 33%, and 54%, respectively ($p_{0.5ng} \leq 0.01$; $p_{1ng} \leq 0.01$; $p_{2ng} \leq 0.01$). After administration of 0.5ng in the long distance experiment, the effect was found to be even more profound, prolonging the median flight time back to the hive by 81% ($p \leq 0.01$). Though, the administration of 0.75ng led to an extended median duration for a flight back to the hive by 101%, this result was not statistically confirmed ($p = 0.188$), probably due to the low number of comparable individuals in this group.

Coumaphos (Flight duration feeder ► hive):
Bees treated with a dose of 5µg coumaphos needed more time for a flight back to the hive immediately after and 24h after oral administration, an effect not found to be persistent after 48 hours (**Fig. 34 C**). The median duration of a homing flight was prolonged by 27% ($p_{5\mu g} \leq 0.01$) immediately after and by 28% ($p_{5\mu g} < 0.05$) 24 hours after treatment. The other two doses used had no significant effect on this phase.

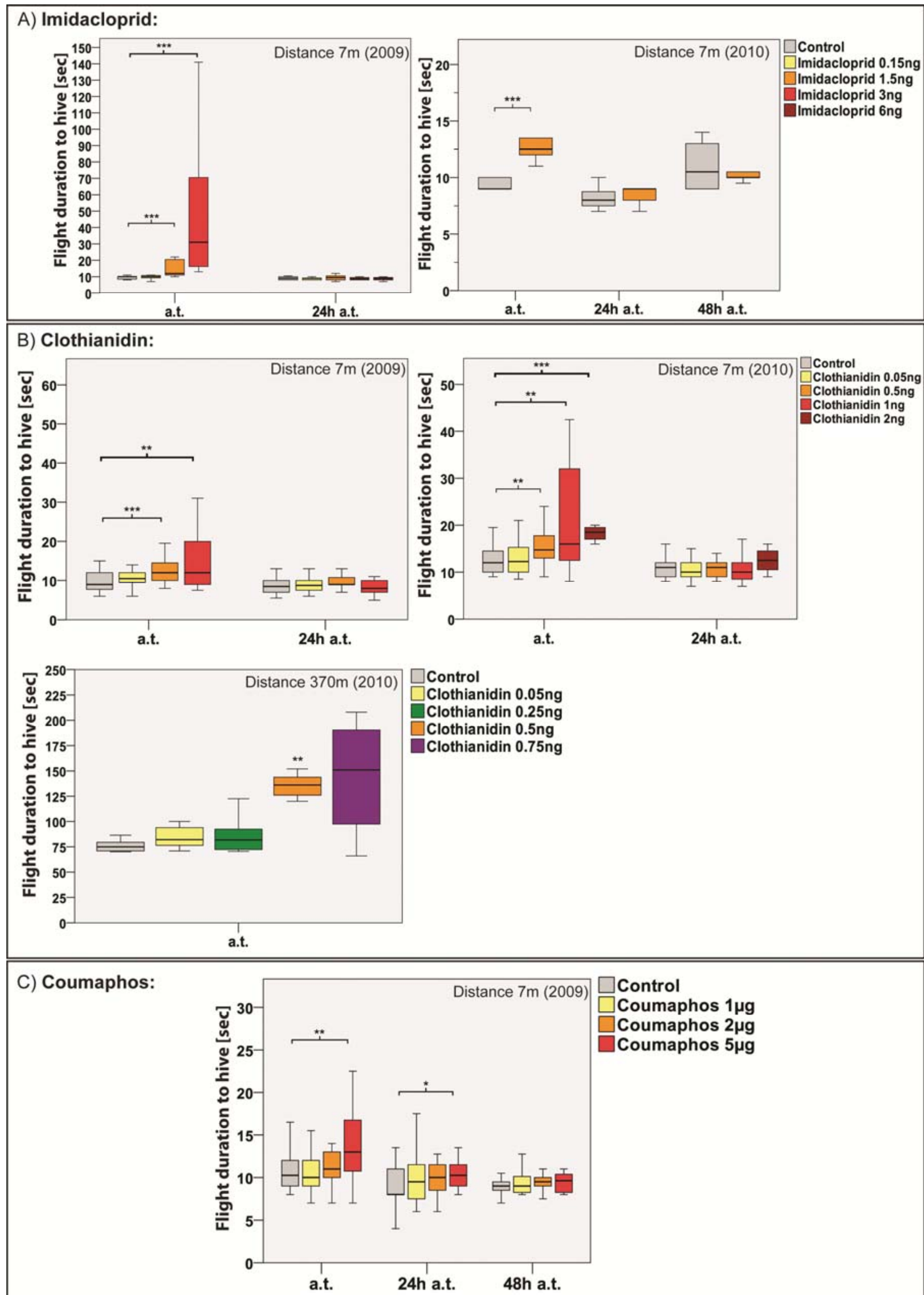

Figure 34: <u>**Effect of imidacloprid (A), clothianidin (B), and coumaphos(C) on the flight duration back to the feeder.**</u> Plotted was the median time in seconds that a bee needed to return from the feeder compartment to the hive during 3-hours observation periods immediately after treatment (a.t.), 24h (24h a.t.), and up to 48h (48h a.t.) after treatment. *= p< 0.05, **= p≤ 0.01, ***= p≤ 0.001. (**A**) Immediately after oral administration of 1.5ng and 3ng imidacloprid a significantly prolonged median flight duration back to the hive compared to the control group was observed. (**B**) Bees treated with 0.5ng , 1ng, and 2ng clothianidin needed significantly longer to fly back to the hive during the three hour observation period immediately after treatment. (**C**) Coumaphos prolonged the flight duration back to the hive at doses of 5µg.The observed effect was found to be persistent only for coumaphos 24h after administration.

VIII. 4.2.3.5 Time interval spent inside the hive between foraging trips

Another factor related to foraging was the time interval from returning to the hive after a foraging trip until leaving the colony again for a subsequent foraging trip. Similar to the foraging trip duration, this parameter was observed during all trials conducted. The median time interval that a bee spent inside the hive during the three-hour observation periods was observed for all substances administered. In addition, since bees in the trials with the neonicotinoids were released at the feeder site, the first time interval spent inside the hive after return was examined more closely. Being released into the colony, the time interval between release and first departure from the hive was determined for bees in the coumaphos trials.

Imidacloprid (Time interval between foraging trips):

The median time that a bee spent within the hive between two foraging trips was significantly prolonged after treatment with 1.5ng and 3ng imidacloprid (**Fig. 35 A**). This effect was not found to be persistent after 24h. In addition, administration of 3ng imidacloprid also significantly affected the first time interval of a bee inside the hive after release at the feeder site. In the experiment with the handheld pen, a tendency towards a longer in-hive stay between foraging trips was observed after the administration of 3ng, which however was not statistically verifiable (p_{3ng}=0.095, 2008). During the following year (2009), 1.5ng and 3ng imidacloprid extended the median time interval of a bee inside the hive by 33% ($p_{1.5ng} \leq 0.001$) and 993% ($p_{3ng} \leq 0.001$, Kruskal-Wallis-Test followed by Mann-Whitney-U-Test), respectively. This was in agreement with the conclusive experiment from 2010, where 1.5ng led to a prolongation of the median in-hive stay by 55% ($p_{1.5ng} \leq 0.01$).

After treatment with 3ng, the first time interval spent within the hive between foraging flights was substantially prolonged by 70% ($p_{3ng} \leq 0.01$, 2008) and 977%, $p_{3ng} \leq 0.001$, 2009), respectively (**Fig. 35 B**). Administration of 1.5ng did not affect the first in-hive stay significantly in either year, even though it was close to significant in 2010 (2009: $p_{1.5ng}$= 0.190; 2010: $p_{1.5ng}$= 0.051).

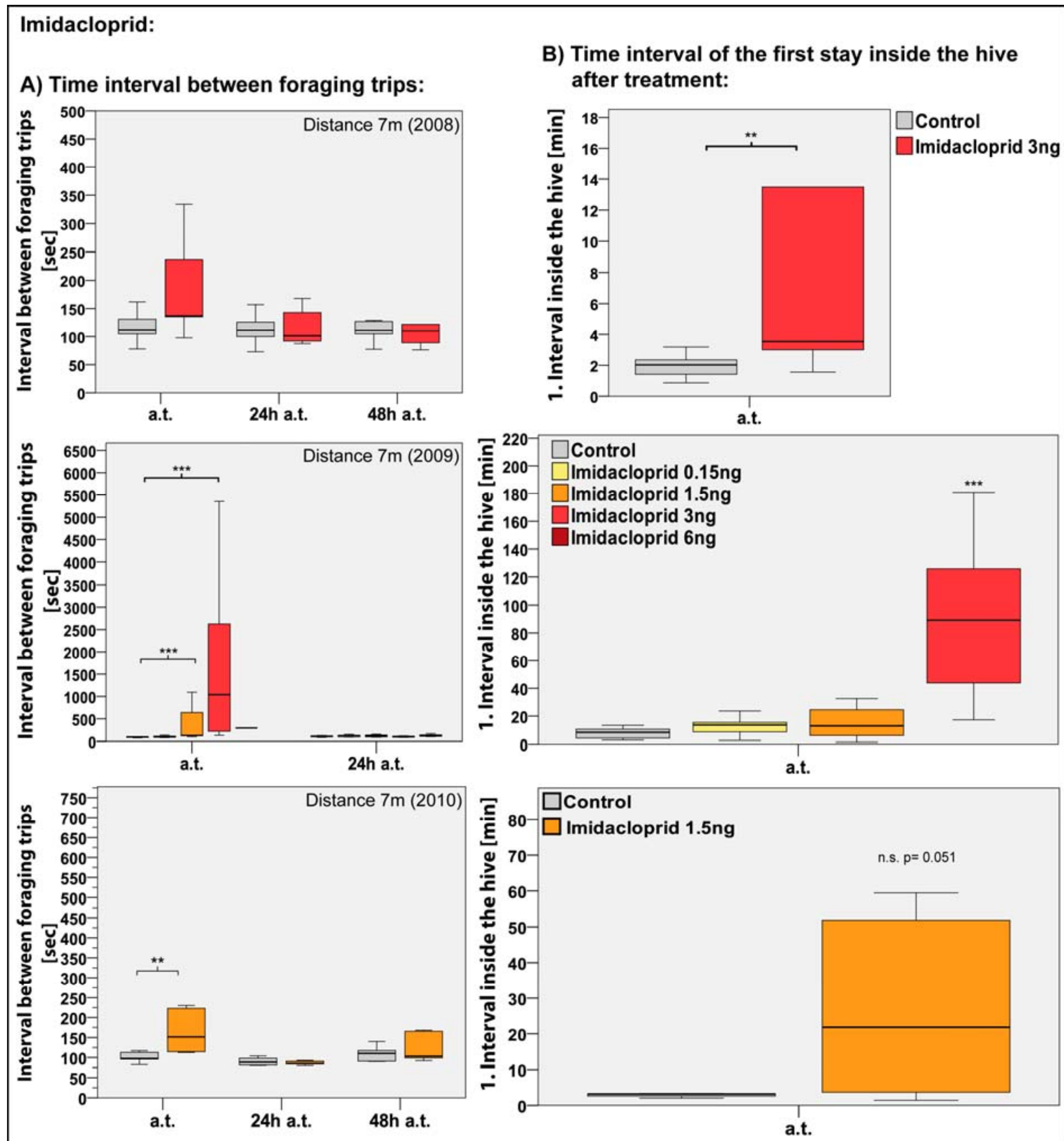

Figure 35 Effect of imidacloprid on the time interval spent inside the hive between foraging trips. Plotted is (**A**) the median period spent inside the hive between two foraging trips (in seconds) during 3-hours observation periods immediately after treatment (a.t.), 24h (24h a.t.), and up to 48h (48h a.t.) and (**B**) the duration of the first in-hive stay (in minutes) for each bee after oral treatment with **imidacloprid** from the experiments conducted over three years.

(A) In the three hour observation period immediately after treatment with 1.5ng in 2009 and 2010 (middle, below), and 3ng imidacloprid in 2009 (middle) these bees needed significantly longer to fly out again after returning from the previous foraging trip compared to the control groups. In 2008 (above) a tendency towards longer in hive stays after treatment was observed but could not be statistically verified.

(B) In addition, administration of 3ng imidacloprid led to a significantly prolonged first stay inside the hive in 2008 and 2009. Administration of 1.5ng prolonged the first in hive stay in 2010 but this difference was not statistically verifiable. *= $p< 0.05$, **= $p\leq 0.01$, ***= $p\leq 0.001$.

Clothianidin (Time interval between foraging trips):

Administration of doses ≥0.5ng clothianidin had a prolonging effect on the median time interval per bee spent inside the hive between foraging trips during the three-hour observation period immediately after treatment (**Fig. 36 A**). This effect did not persist after 24 hours. The first in-hive stay after being released at the feeder was found to be prolonged by 1ng in all experiments.

The time interval of in-hive stays between foraging trips per bee was prolonged by 16% for 0.5ng ($p_{0.5ng} < 0.05$) and 27% for 1ng ($p_{1ng} < 0.05$) in 2009. In a repetition of these short-distance experiments in the following year, it was observed that only oral administration of 1ng prolonged the time interval by 43% ($p_{0.5ng} < 0.05$). Nevertheless, during the long distance experiment, bees treated with 0.5ng and 0.75ng spent 89% ($p_{0.5ng} \leq 0.001$) and 139% ($p_{0.75ng} \leq 0.01$) more time inside the hive between foraging trips, respectively.

In the first clothianidin experiments conducted in 2009, the first interval inside the hive was found to be prolonged by 47% after treatment with 0.5ng ($p_{0.5ng} < 0.05$) and by 307% after treatment with 1ng ($p_{1ng} \leq 0.001$) (**Fig. 36 B**). During the short-distance experiments in 2010, only administration of 1ng and 2ng prolonged the first interval by 132% ($p_{1ng} \leq 0.001$) and 882% ($p_{2ng} \leq 0.001$) respectively. Administration of 0.5ng clothianidin during the long distance experiment, prolonged the median duration for the first interval inside the hive by 151%. Probably due to the large variation of this phase in this treatment group together with the relatively low number of testable bees (n= 8), this difference was not found to be statistically significant (p= 0.211).

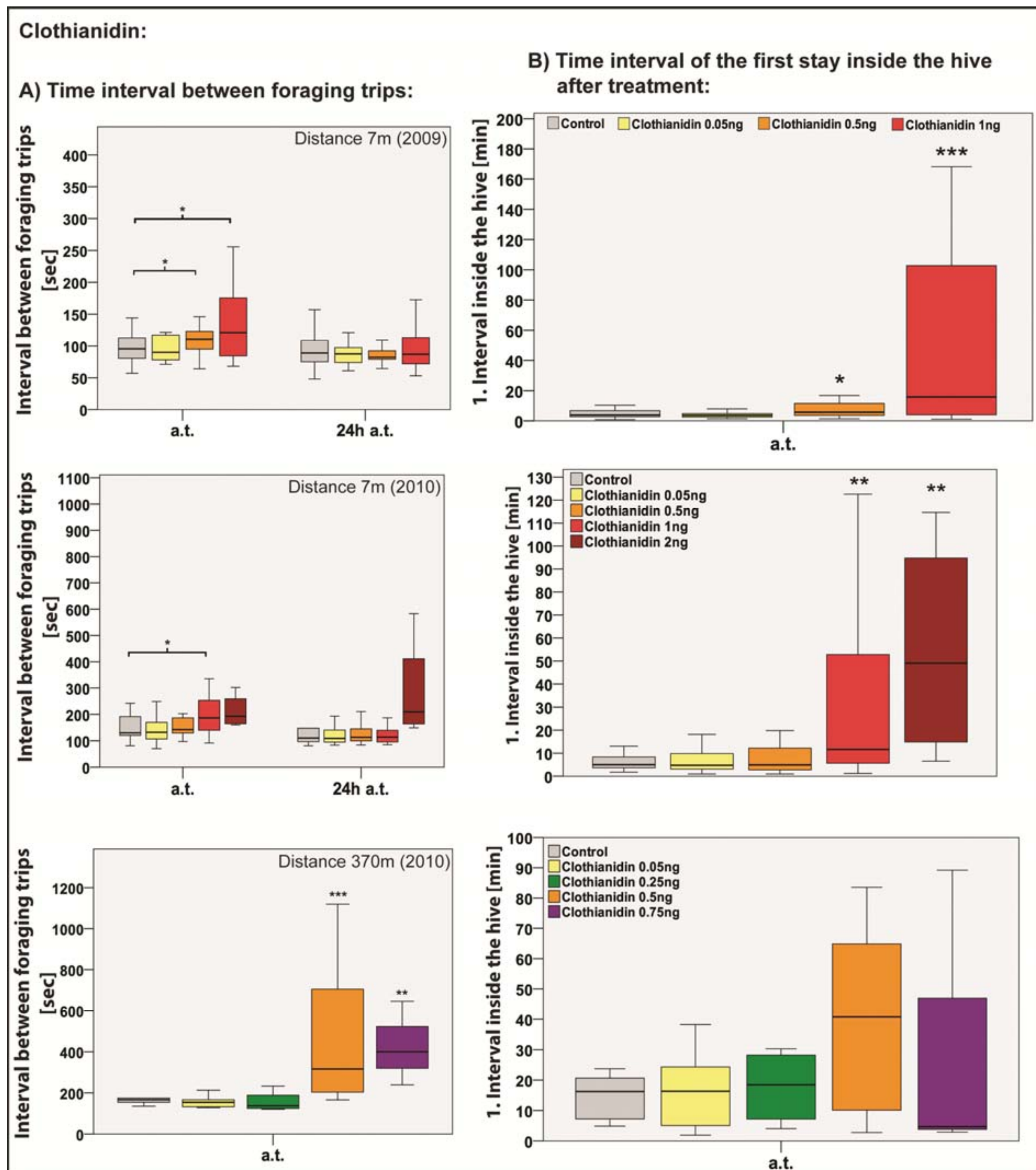

Figure 36 **Effect of clothianidin on the time interval spent inside the hive between foraging trips.** Plotted is (**A**) the median time period spent inside the hive between two foraging trips (in seconds) during 3-hours observation periods immediately after treatment (a.t.), 24h (24h a.t.), and up to 48h (48h a.t.) and (**B**) the duration of the first in-hive stay (in minutes) for each bee after oral treatment with **clothianidin** from the experiments conducted over two years.

(**A**) Doses ≥0.5ng clothianidin were found to affect the median time interval inside the hive between foraging trips in the shorts distance experiments in 2009 (above) and the long distance experiment in 2010 (below), while only doses of 1ng significantly altered this parameter in the short distance experiments in 2010 (middle).

(**B**) The duration of the first stay inside the hive immediately after treatment with 1ng was significantly prolonged in 2009 (above) and 2010 (middle), while 0.5ng affected this parameter in 2009 (above) only. During the long distance experiment in 2010 (below) treatment with 0.5ng appeared to prolong the duration of the first stay inside the hive, though the difference was not statistically verifiable. *= p< 0.05, **= p≤ 0.01, ***= p≤ 0.001.

Coumaphos (Time interval between foraging trips):

The median time intervals per bee spent inside the hive were prolonged significantly after administration of 2µg and 5µg coumaphos (**Fig. 37 A**). This effect was still detectable 24h after treatment with 2µg and up to 48h after treatment with 5µg. During the experiments in 2008, the median time intervals spent inside the hive between foraging trips for bees treated with 5µg was extended by 495% immediately ($p_{5µg\ a.t.} \leq 0.001$), by 588% twenty-four hours ($p_{5µg\ 24ha.t.} \leq 0.001$), and by 347% forty-eight hours ($p_{5µg\ 48ha.t.} \leq 0.001$) after treatment. Bees that were fed with 2µg spent 24% ($p_{2µg\ a.t.} < 0.05$) and 50% ($p_{2µg\ 24ha.t.} \leq 0.01$) more time inside the hive between foraging trips immediately and 24 hours after treatment. In the following year, 2µg and 5µg prolonged the median time interval spent inside the hive by 19% ($p_{2µg} < 0.05$) and by 95% ($p_{5µg} < 0.001$), respectively. This adverse effect still persisted for bees treated with both dosages 24h after treatment (2µg: +20%, $p < 0.05$; 5µg: +13%, $p < 0.05$) but could not be statistically verified after 48h (2µg: +11.5%, $p = 0.087$; 5µg: +17%, $p = 0.108$). Administration of 1µg had no effect on this parameter in either year (see also **Fig. S3**, Supporting Information)

In contrast to the treatment with the neonicotinoids, bees were released into the colony after administration of coumaphos. Therefore, the time interval between release into the hive and initial registration at the hive entrance was examined for these bees. This period was significantly prolonged after administration of 5µg regardless of the experimental year (**Fig. 37 B**). Bees treated with 2µg needed significantly longer to leave the hive again after release in 2008, which was not found though in 2009. The median duration before the initial registration at the hive entrance was prolonged 150% and 90% after administration of 5µg and 2µg, respectively ($p_{5µg} \leq 0.001$, $p_{2µg} \leq 0.01$; Mann-Whitney-U-Test, **Fig. 37 B**) in 2008. Treatment with 5µg extended the period before the initial registration at the hive entrance by 181% in the 2009 ($p_{5µg} < 0.05$). The administration of the 1µg coumaphos had no significant effect on this parameter (see also **Fig. S4**, Supporting Information).

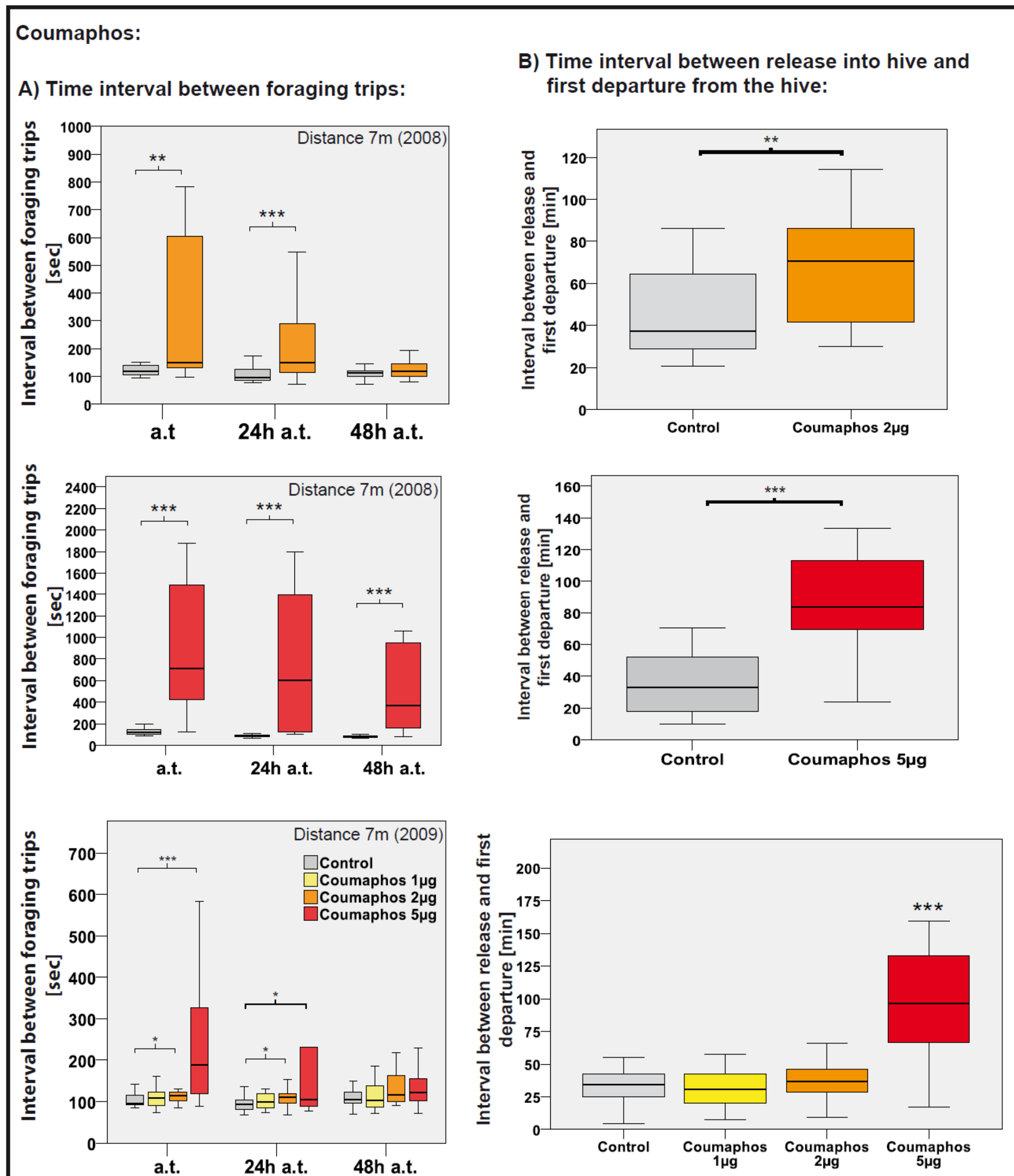

Figure 37: Effects of coumaphos on the time interval spent inside the hive between foraging trips. Plotted is (**A**) the median period each bee spent inside the hive between foraging trips (in seconds) during 3-hours observation periods immediately after treatment (a.t.), 24h (24h a.t.), and up to 48h (48h a.t.) and (**B**) the duration of the first in-hive stay (in minutes) for each bee after oral treatment with **coumaphos** from the experiments conducted over two years. *= p< 0.05, **= p≤ 0.01, ***= p≤ 0.001.
(A) Oral administration of 2µg and 5µg coumaphos significantly prolonged the median time interval between foraging trips up to 24h and 48h after treatment, respectively in 2008 (above, middle) and up to 24h for both doses in 2009 (below).
(B) In both experimental years (middle, below) treatment with 5µg coumaphos significantly prolonged the time interval from release into the hive until first departure from the hive, while administration of 2µg significantly prolonged this parameter in 2008 only (above).

The alterations of the different foraging trip phases in percent for the observation period immediately after administration of the different substances are summarized in **Table 11**.

Table 12: Summary of alterations of foraging trip phases immediately after administration of imidacloprid, clothianidin, and coumaphos over consecutive years compared to the control. Positive percentage values and arrows pointing upward represent prolongations, negative values and arrows pointing downward represent reductions. yellow background = statistically significant difference compared to the control group; red background = no data available. *= p< 0.05, **= p≤ 0.01, ***= p≤ 0.001.

	Distance feeder: 7 m	a.t. 2009	Distance feeder: 7 m	a.t. 2009	a.t. 2010	Distance feeder: 370 m	a.t. 2010	Distance feeder: 7 m	a.t. 2009
Duration Feeder►Hive	Imida 0.15ng	↑ 15%	Clothia 0.05ng	↓* -28%	↓ -5%	Clothia 0.05	↑ 4%	Couma 1µg	↓ -14%
	Imida 1.5ng	↑** 65%	Clothia 0.5ng	↓ -9%	↑ 5%	Clothia 0.25	↑ 10%	Couma 2µg	↓ 16%
	Imida 3ng	↑*** 241%	Clothia 1ng	↑ 14%	↑ 10%	Clothia 0.5	↑** 17%	Couma 5µg	↑ 12%
	Imida 6ng		Clothia 2ng		↑ 31%	Clothia 0.75	↑** 50%		
Duration of stay at feeder	Imida 0.15ng	↑ 6%	Clothia 0.05ng	↓ -5%	↓ -2%	Clothia 0.05	↑ 5%	Couma 1µg	↓ -3%
	Imida 1.5ng	↑* 28%	Clothia 0.5ng	↑ 4%	↑* 17%	Clothia 0.25	↑ 12%	Couma 2µg	↑ 7%
	Imida 3ng	↑* 46%	Clothia 1ng	↑*** 22%	↑*** 39%	Clothia 0.5	↑*** 55%	Couma 5µg	↑*** 14%
	Imida 6ng		Clothia 2ng		↑*** 71%	Clothia 0.75	↑** 45%		
Duration Hive►Feeder	Imida 0.15ng	0	Clothia 0.05ng	↑ 17	↑ 2	Clothia 0.05	↑ 9%	Couma 1µg	↓ -3%
	Imida 1.5ng	↑** 20%	Clothia 0.5ng	↑*** 33%	↑** 23%	Clothia 0.25	↑ 9%	Couma 2µg	↑ 7%
	Imida 3ng	↑*** 210%	Clothia 1ng	↑** 33%	↑** 33%	Clothia 0.5	↑** 81%	Couma 5µg	↑** 27%
	Imida 6ng		Clothia 2ng		↑** 54%	Clothia 0.75	↑ 101%		
Time interval between foraging trips	Imida 0.15ng	↑ 7%	Clothia 0.05ng	↓ -6%	↑ 2%	Clothia 0.05	↓ 8%	Couma 1µg	↑ 14%
	Imida 1.5ng	↑*** 33%	Clothia 0.5ng	↑* 16%	↑ 10%	Clothia 0.25	↓ 18%	Couma 2µg	↑* 19%
	Imida 3ng	↑*** 993%	Clothia 1ng	↑* 27%	↑* 43%	Clothia 0.5	↑*** 89%	Couma 5µg	↑*** 95%
	Imida 6ng		Clothia 2ng		↑ 48%	Clothia 0.75	↑** 139%		
1. stay inside the hive a.t.	Imida 0.15ng	↑ 64%	Clothia 0.05ng	↓ -6%	↓ -5%	Clothia 0.05	↑ 1%		
	Imida 1.5ng	↑ 56%	Clothia 0.5ng	↑* 47%	↓ 2%	Clothia 0.25	↑ 14%		
	Imida 3ng	↑*** 977%	Clothia 1ng	↑*** 307%	↑** 132%	Clothia 0.5	↑ 151%		
	Imida 6ng		Clothia 2ng		↑** 882%	Clothia 0.75	↓ -71%		
Time Interval between release into and first departure from the hive a.t.								Couma 1µg	↓ -11%
								Couma 2µg	↑ 6%
								Couma 5µg	↑* 181%

VIII. 5. Discussion

Our study used the RFID-technology to analyse the impact of insecticide compounds on honeybee foraging behavior. Decourtye already showed that fipronil at doses of 0.3ng/bee reduced the number of foraging flights to the feeder and prolonged the duration of the homing flight (Decourtye et al. 2011). These effects were observable for up to three days. Similar effects were found for the compounds used in this study. In contrast to Decourtye, who conducted his tests under semi-field conditions, the described experiments were conducted under field conditions, allowing the test colonies to normally provide themselves with necessary additional resources including pollen, water, and propolis. Furthermore, since the test is designed to detect effects on individual bees, the tested bees were fed defined amounts of the pesticide individually with the differently spiked sugar solutions instead of bulk feeding them in a cage. Nevertheless, by independentl developingy a similar approach, this considerably strengthens the validity of the method. In particular, it could show that the method is applicable not only under semi-field- but also under field conditions
By obtaining quantifiable and accurate data about a minimum of six different parameters of foraging behavior, sub-lethal effects for all three tested substances, imidacloprid, clothianidin, coumaphos, used in this study. RFID-tracking allowed for simultaneous testing of multiple groups of bees, each group treated with a different dosage of a tested insecticide compound. The additional control group made the experiments independent from altering environmental conditions.

Impairments after administration of imidacloprid were detected at doses of 1.5ng per bee, which would equal a concentration of around 115 ppb (parts per billion) in nectar. These results are in agreement with previous studies, which tested the effect of imidacloprid on homing and foraging behavior (Bortolotti et al. 2003). Yang and colleagues (Yang et al. 2008) found effects on foraging behavior at concentrations as low as 50µg/L (40-50ppb). These concentrations documented are still more than twenty-five to fifty times higher than the residues found in nectar of sunflowers (*Helianthus*, 1.9ppb) (Schmuck et al. 2001). Treatment with the lowest dose of imidacloprid (0.15ng; 11.5 ppb), which is about six-fold higher than any residues found in nectar, had no recognizable effect on foraging behavior after one-time administration. Nevertheless, bees may be exposed to almost 100-fold higher doses

than tested in our experiments, as shown in a study concerning the insecticide contamination of guttation drops, xylem fluids that are excreted at the leaf margins (Girolami et al. 2009) in seed dressed crops. It remains unclear though, if water foragers collect these contaminated fluids in the field, and how they are affected by them if collected.

This is to my knowledge the first study on foraging behavior of honeybees that presents sub-lethal effects after acute oral treatment with clothianidin. Dosages of 0.5ng (38ppb) negatively influence the foraging behavior and low dosages (0.05ng; 3.8ppb) had effects on certain components of a foraging trip even though they did not seem to affect the foraging activity or the total foraging trip duration. Clothianidin elicited detrimental sub-lethal effects at somewhat lower doses (0.5ng/bee) compared to imidacloprid (1.5ng/bee). Bees started to disappear at levels of ≥1ng for clothianidin, while we could register the first bee losses for imidacloprid at doses ≥3ng. This indicates a stronger impact of clothianidin compared to imidacloprid, which is in agreement with previous reports that both oral (Girolami et al. 2009) and contact toxicity (J. Bailey et al. 2005) levels are lower for clothianidin. Both neonicotinoid insecticides are known to be partial agonists for different types of the insect nicotinic acetylcholine receptor (nAChR) (S. Buckingham et al. 1997; Déglise, B. Grünewald, and M. Gauthier 2002; Tomizawa, Lee, and Casida 2000; Tomizawa and Casida 2003; Tomizawa and Casida 2005b). In vitro experiments that observed the action of imidacloprid and clothianidin on native nAChRs of cholinergic neurons from *Drosophila* 3rd instar larvae (Brown et al. 2006) and nAChRs of the terminal abdominal ganglion neurons of the American cockroach (Ihara et al. 2006) showed greater agonist efficacy of clothianidin compared to imidacloprid. A similar effect on cholinergic neurons in adult honeybees could be an explanation for this finding that clothianidin elicits detrimental effects at lower doses than imidacloprid. In general, both neonicotinoid substances led to similar effects on the observed foraging parameters. The only difference was found when investigating the flight time to the feeder. Bees treated with imidacloprid needed more time to fly to the feeder while no effect on this phase was observed after administration of clothianidin. Relating this to the symptoms observed after administration of higher doses of both substances it could be another indication for differences in their efficacy. Higher doses of imidacloprid (≥3ng) led to reduced movement, eventually leading to immobility and trembling movements, which is in accordance with previously reported doses of ≥

2.5ng by Lambin et al. (Lambin et al. 2001). This might have occurred to some degree in the lower doses as well, but escaped visual observation. Higher doses of clothianidin (≥1ng), in contrast resulted in an arched abdomen, which did not reduce the mobility of the bees like imidacloprid did. Girolami et al. (Girolami et al. 2009) reported that when their abdomens were arched, the bees still retained their flying capability. Taking this into consideration, it could explain why the detrimental effect on flight behavior by clothianidin was less pronounced compared to imidacloprid, though still significant compared to the controls. Furthermore, it might be hypothesized that imidacloprid and clothianidin aim at differing targets, i.e. different subtypes of receptors located on pre-motoneurons and motoneurons of honeybees, though their cell physiological actions are still unknown. In this context, a study by Thany (Thany 2009) on DUM-(dorsal unpaired median) neurons isolated from the cockroach *Periplaneta Americana* showed that clothianidin acted on imidacloprid-sensitive and -insensitive nAChR subtypes. DUM-neurons are known for their neuromodulatory role in altering the performance of motor patterns and are thought to be homologous to VUM (ventral unpaired median)-neurons (Bräunig and Pflügler 2001; Schröter, Malun, and Menzel 2007) of honeybees because of their similar morphology and physiology.

The lowest dose used (0.05ng) significantly reduced the median flight time per bee to the feeder in 2009 though not in 2010 and was found to shorten the duration of foraging trips and especially the time spent at the feeder 24 hours after administration compared to the control group. This might indicate an increase motor activity. This proposed phenomenon of increased motor activity by honeybees after neonicotinoid exposure was already reported in another study after topical application of 1.25ng imidacloprid (Lambin et al. 2001). In contrast to this, the dosages used for imidacloprid in our study did not seem to increase motor activity.

Both substances led to a longer 1st and 2nd period inside the hive before returning to the feeder. This is likely due to a prevailing toxic effect on the bees while they were inside the hive. They stayed in the hive until the effect ceased and they were able to fly out again. This is consistent with the fact that bees that did not return to the hive after treatment were not registered again, but the majority of bees that made it back to the hive later returned to the feeder.

Tests with the organophosphate coumaphos during the summers of 2008 and 2009, showed high doses are needed to cause alterations in the foraging behavior. Effects

were regularly observed after treatment with the highest administered dose of 5µg per bee and especially in the first year after administration of 2µg. To put this into perspective: During the course of a standard Perizin® application, an amount of 32mg coumaphos (1ml Perizin®) in 50ml water solution is administered into a normally populated hive (package insert Perizin®), equaling a dose of 640ng/µl. Assuming that 50000µl solution ís applied to a colony of 50000 bees this would mean that if every bee takes up a microliter of this solution, each bee might take up 0.64µg of coumaphos. To reach the doses of coumaphos that were administered in this experiment a single bee would need to take up either 1.56µL (1µg), 3.125µL (2µg), or 7.8125µL (5µg) of the solution. Taking into consideration that Perizin® is administered in a watery solution and since coumaphos was found to act topically on the varroa mite rather than orally through the haemolymph of the bee, it is rather improbable that a bee would take up greater amounts of the solution orally. Thus, one can assume that bees do not take up much more than 1µg coumaphos orally during a standard *Varroa* treatment with Perizin®. In either experimental year a dose of 1µg coumaphos per bee did not affect the behavior of the bee in any way.

Another exposure route to coumaphos besides Perizin® treatment are the residues that occur in bee products (wax, pollen, honey). Coumaphos is a fat-soluble substance is known to contaminate the wax. From here it migrates into the honey (Wallner 1992). Higher concentrations in the wax lead to higher concentrations in the stored honey. Since there is no natural degradation of varroacides in the wax, multiple applications can lead to an accumulation of these substances (Wallner 1999). Kochansky and colleagues (Kochansky, Wilzer, and Feldlaufer 2001) also showed that higher contaminations of the wax lead to higher residues in the honey with concentrations of 1000ppm (mg/kg) leading to up to 400ppb (µg/kg) in the honey. Usually, determined residue concentrations found in the honey did not exceed 15 ppb (E. Klein et al. 1986; Wachendörfer & Keding 1988; Thrasyvoulou & Pappas 1988 as reviewed in Wallner 1999). Thus, a bee would need to take up more than 50 ml of contaminated honey, neglecting possible detoxification mechanisms, for a dose of 1µg coumaphos. Considering this, the only way bees could take up the tested amounts of coumaphos would be during a Perizin® application.

The highest dose of 5µg coumaphos per bee, ranging in the area of the oral LD_{50} of 3-6µg reported by van Buren and colleagues (van Buren et al. 1992), did not so much cause mortality but strongly affected foraging by reducing the activity of the

treated bees over a period of up to 48h after treatment in comparison to the control. Such a lingering effect on foraging activity was also observed after administration of 2µg in 2008, with a similar though insignificant trend found in 2009. This indicates that if bees are affected by the substance they need longer to recover from these effects. On the one hand, this could be due to the different modes of action of organophosphates and neonicotinoids, acting on the acetyl choline esterase and the nicotinic acetyl choline receptor, respectively. Another explanation could lie in different mechanisms for detoxification of the honeybee. A study by Claudianos and colleagues (Claudianos et al. 2006) investigated the existence of different genes coding for detoxification enzymes in *Drosophila*, *Anopheles gambiae*, and *Apis mellifera* and found that the honeybee has a deficit of detoxification enzymes. Interestingly though, genes from an enzyme family related with pyrethroid and neonicotinoid resistance (CYP6- and CYP9- Cytochrome P450 monooxygenase) when overexpressed in *Anopheles gambiae* (CYP6Z1) (Nikou, Hilary Ranson, and Janet Hemingway 2003), *Musca Domestica* (CYP6D1, CYP6D3) (Kasai and Scott 2001) and *Drosophila melanogaster* (CYP6G1) (Daborn et al. 2002) are also present in ample numbers in the honeybee. Meanwhile, it was shown by Claudianos (Claudianos et al. 2006) that honeybees have a deficit of the insect-specific Delta and Epsilon classes of the Glutathione-S-transferase (GST). Especially those classes of GSTs are considered to play a role in insecticide metabolism of organochlorines like DDT (Tang and Tu 1994; H Ranson et al. 2001; Lumjuan et al. 2005) and organophosphates like parathion, methyl-parathion, and paraoxon (Dauterman 1985; F M Chiang and Sun 1993; Huang et al. 1998; Wei, Clark, and Syvanen 2001). On the other hand, van Buren and colleagues (van Buren et al. 1992) proposed that there could be some sort of detoxification mechanism for coumaphos in the honeybee as well, after conducting a chronical toxicity test, feeding 21µg coumaphos/bee over 7 days before 50% mortality was reached. Li and co-workers (Li et al. 2003) reported that enhanced cytochrome P450 monooxygenase activity is present in coumaphos-resistant strains of the southern cattle tick, *Boophilus microplus*, and a study by Johnson and colleagues (Johnson et al. 2009) showed that detoxification through cyt P450 monooxgenases was also involved in honeybees. Nevertheless, the results presented here point into the direction that honeybees recover faster from the effects of a sub-lethal dose of imidacloprid and clothianidin, compared to a sublethal dose of coumaphos. To effectively test this hypothesis of a

better detoxification mechanism by honeybees towards neonicotinoids rather than to organophosphates like coumaphos, one option could be a chronical administration of low doses, a scenario much more likely since bees might be repeatedly exposed to low doses while foraging from seed dressed plants.

In conclusion, radiofrequency identification provides a considerable help concerning risk assessment of insecticides on a sub-lethal level. Subsequent to initial mortality tests to determine the LD_{50} of an insecticide, the RFID-method could be used in field-like tests to investigate possible effects of doses thought to be non-hazardous for honeybees.

IX. Final Conclusion

In summary, I think it is appropriate to state that with RFID-tracking it is possible to retrieve reliable data about the influence of different potential stress factors on honeybees. The results emphasize that stress factors as diseases, thermal stress, or insecticides can have similar effects in terms of shortened lifespan (*Nosema*, sub-, above-optimal temperatures), precocious activity *(Nosema*, above-optimal temperatures), and increased foraging trip and flight durations (insecticides, above-optimal temperatures, *Nosema*). Therefore, the basic behavioral mechanisms with which bees react to these different stressors might be similar and could serve as a starting point to increase this reaction, simultaneously increasing the health and resistance of the colonies.

In addition, a basis is provided for subsequent investigations concerning the effects of a chronical exposure of honeybees to low insecticide doses as occurring by regular agricultural practices. Furthermore, hypotheses can be tested that instead of single factors interactions between different stress factors are playing a role in decline of bee colonies worldwide (Vidau et al. 2011; Alaux et al. 2010). I am confident that radiofrequency identification of honeybees will prove quite helpful in the respective fields of research.

X. References

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XI. Curriculum vitae

Christof Wolfgang Schneider

Date of Birth:	17.09.1979
Place of Birth:	Heidelberg
Nationality:	German

Education:

Oct. 2010 – March 2011	**Julius Maximilians-University, Würzburg, Germany** Research assistant of Professor Jürgen Tautz at the department of "Behavioral Physiology and Sociobiology"
Oct. 2007 – Sept. 2010	**Johann-Wolfgang-Goethe University of Frankfurt/Main, Germany** PhD studies at the Institut für Bienenkunde, Oberursel, Germany Topic of the PhD Thesis "Detecting the influence of different stresses on honeybee foragers at the hive entrance using Radiofrequency Identification " Supervised by Prof. Jürgen Tautz and PD Dr. Stefan Fuchs
Oct. 2004 – Sept. 2007	**Julius-Maximilians-University, Würzburg, Germany** Studies in Biology Graduation with Diploma Topic of diploma thesis „Der Austrag der Varroamilbe, *Varroa destructor*, durch Honigbienenarbeiterinnen als möglicher Resistenzfaktor." supervised by PD Dr. Stefan Fuchs
Oct. 2001 – Sept. 2004	**University of Technology, Darmstadt, Germany** Studies in Biology Successful intermediate examination
Oct. 2000 – Sept. 2001	**University of the Saarland, Saarbrücken, Germany** Academic studies of Business Administration, without graduation
Oct. 1999 – Sept. 2000	Alternative Civilian Service at the „Akademie Frankenwarte", Würzburg, Germany
Sept. 1991 – July 1999	**Siebold – Gymnasium, Würzburg, Germany** General Secondary Education Successful completion of the Final Examination (Abitur)
Sept. 1990 – July 1991	**St. Anna – Gymnasium, München, Germany** First year of Secondary School

XII. Publications

In preparation:

Schneider, C.W., Tautz, J., Grünewald, B., Fuchs, S., *Determination of sublethal insecticide effects on foraging behavior of the honeybee using radiofrequency identification.*

Kralj, J., Schneider, C.W., Grünewald, B., Fuchs, S., *Monitoring disease-induced behavioral changes in forager honeybees Apis mellifera at the colony entrance.*

XIII. Danksagungen

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XIV. Supporting Information:

I. Figures

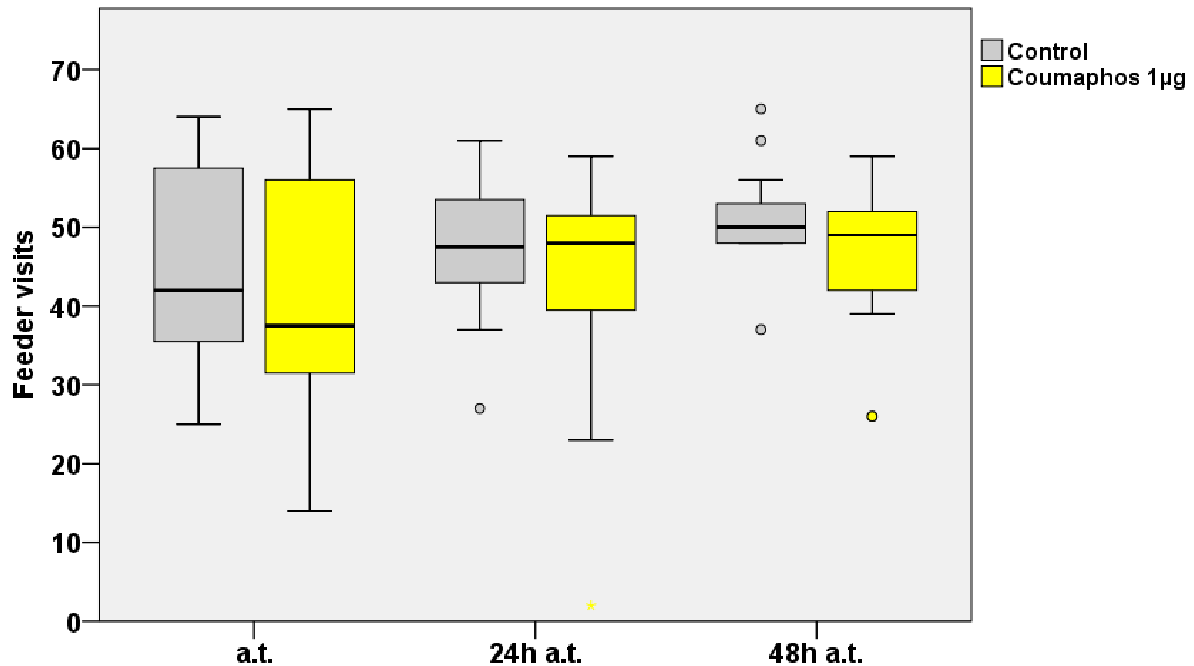

Abbildung S1 **Influence of 1µg coumaphos on the the number of feeder visits.** Ordinate: Number of feeder visits. Abscissa: different observation periods. a.t.= immidiately after treatment, 24h a.t.= 24h after treatment, 48h a.t.= 48h after treatment.

N= Number of bees treated bees returning to the feeder, %= proportion of treated bees returning to the feeder.

	a.t.	24h a.t.	48h a.t.
Control	N= 20 (100%)	20 (100%)	17 (85%)
Coumaphos	N= 20 (100%)	19 (95%)	16 (80%)

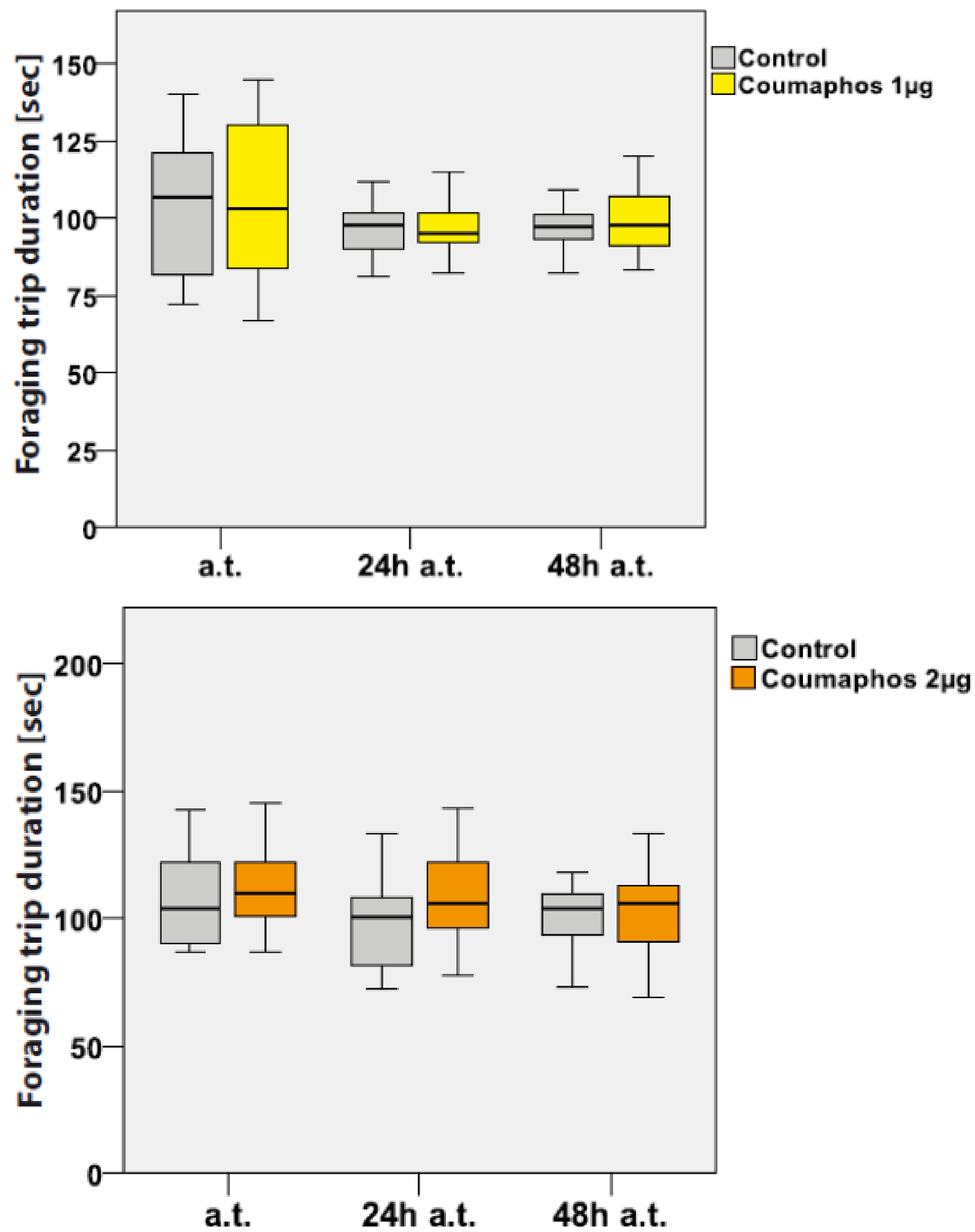

Figure S2 **Duration for a foraging trip after treatment 1µg (above) and 2µg coumaphos (below).** Ordinate: Median time in minutes each bee needed for a foraging trip. Abscissa: different observation periods. a.t.= immideately after treatment, 24h a.t.= 24h after treatment, 48h a.t.= 48h after treatment.

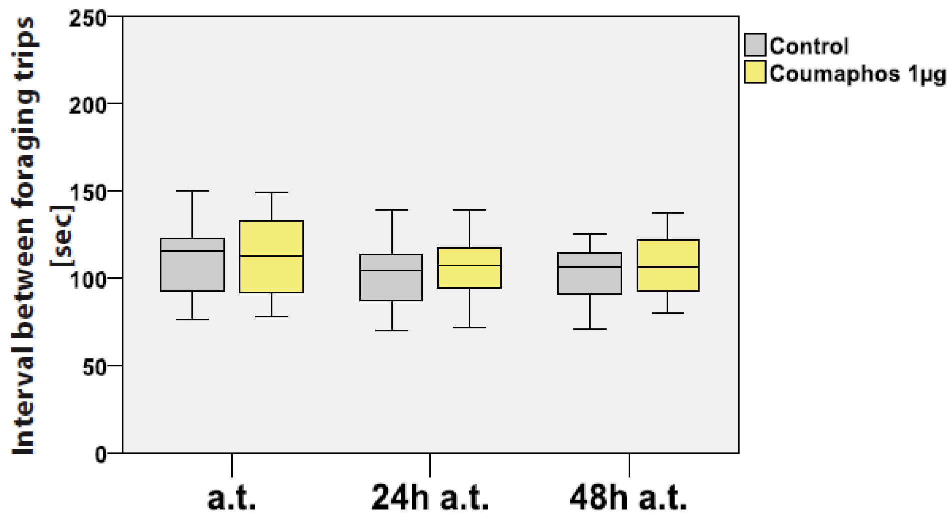

Abbildung S3: **Duration for a foraging trip durations after treatment with 1µg coumaphos.** Ordinate: Median time interval a bee spent inside of the hive in minutes. Abscissa: different observation periods. a.t.= immideately after treatment, 24h a.t.= 24h after treatment, 48h a.t.= 48h after treatment.

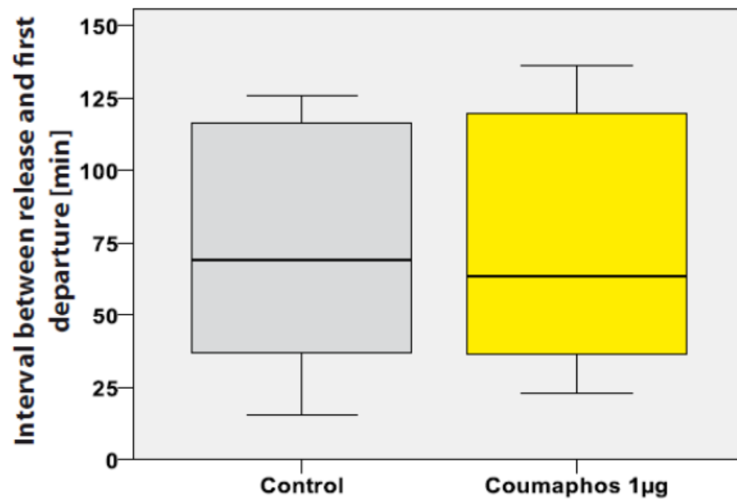

Abbildung S4: **Effects of coumaphos on the time interval spent inside of the hive between foraging trips** Ordinate: Time in minutes each bee needed from being released into the hive until being registered again at the hive entrance. Abscissa: treatment.

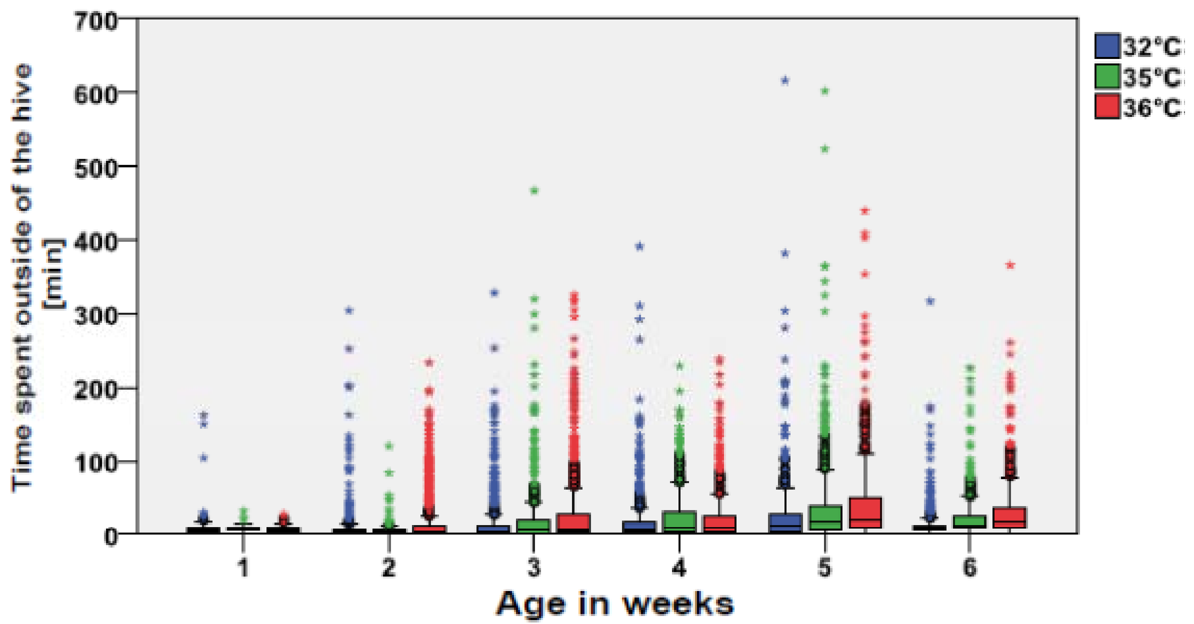

Figure S5: Comparing the period of time bees of the three temperature groups spent outside of the hive per week of life. Ordinate: Median time a bee spent outside of the hive in minutes per week. Abscissa: Age of the bees in weeks. Diagram includes Median + 25th and 75th percentiles + whiskers + outliers (o= outliers, asterisk (*)= extreme outliers).

II. Tables

Tabelle S 1: Mortality rate over 48h during the different toxicity tests conducted for clothianidin

Cage #	Test #	Dose[ng/bee]	N (bees treated)	Mortality 24h	Mortality 48h
1	1	Control	10	0	4
2	1	Control	10	0	2
3	2	Control	10	0	0
4	2	Control	10	0	2
5	3	Control	9	1	no observation
6	3	Control	10	0	no observation
7	4	Control	10	1	3
8	4	Control	10	1	2
9	4	Control	10	0	1
10	1	0,15	10	0	3
11	1	0,15	10	0	2
12	2	0,15	10	1	3
13	2	0,15	10	0	0
14	3	0,15	10	0	no observation
15	3	0,15	9	0	no observation
16	4	0,15	10	1	3
17	4	0,15	10	0	3
18	4	0,15	10	0	1
19	1	1,5	10	1	3
20	1	1,5	10	0	2
21	2	1,5	10	4	5
22	2	1,5	10	1	2
23	3	1,5	10	0	no observation
24	3	1,5	10	1	no observation
25	3	1,5	10	1	no observation
26	4	1,5	10	2	7
27	4	1,5	10	0	2
28	4	1,5	10	2	4
29	1	3	10	4	7
30	1	3	10	6	7
31	2	3	10	9	9
32	2	3	10	4	5
33	3	3	10	10	no observation
34	3	3	10	7	no observation
35	3	3	10	3	no observation
36	4	3	10	9	9
37	4	3	10	7	8
38	4	3	10	7	7
39	1	6	10	9	9

40	1	6	11	8	9
41	2	6	10	9	9
42	2	6	10	10	10
43	3	6	10	10	no observation
44	3	6	10	10	no observation
45	3	6	9	9	no observation
46	4	6	10	10	10
47	4	6	10	10	10
48	4	6	10	10	10

Table S 2: Measurements of the different Plexiglas® base structures

	Plexiglas® base structure	
	old	new
total width (mm)	70	92
width wall to wall	52	77
total length	260	260
total height	43	43
height floor to ceiling	30	30
ø opening for dead bee receptacle	55	55

III. Syntax-Files

Explanations for the different syntax are written in blue while the syntax commands are written in black.

1. "Number syntax":

Sorts data in descending fashion and creates a variable named "Number" which contains only the last five digits of the ID Number which are usually different in all other TAG-IDs. The Number variable has to be transformed by hand from "String" value into a "Numeric" value. This is done in "Variablen Ansicht" on the lower left area of the data sheet.

```
Sort cases by
date (d) time (d).
STRING Number (A10).
COMPUTE Number = SUBSTR(Tag,16,5).
EXECUTE.
```

……………………………………………………………………………………………………..

2. Antenna-Filter-Syntax:

Sorting data in the fashion that the "Number Syntax" does is needed for the subsequent "Antenna-Filter-Syntax": it erases all unnecessary antenna counts from the same antenna, e.g. when a bee gets caught under one antenna due to oncoming traffic, in a time window of two seconds after the initial one.

```
Sort cases by
Number (d) date (d) time (d).
USE ALL.
COMPUTE filter_$=(tag<>lag(tag) | antenna<>lag(antenna)  |
Scanner_ID<>lag(Scanner_ID) | time<lag(time)-2).
VARIABLE LABEL filter_$ 'tag<>lag(tag) | antenna<>lag(antenna)  |
Scanner_ID<>lag(Scanner_ID) | time<lag(time)-2 (FILTER)'.
VALUE LABELS filter_$  0 'Nicht ausgewählt' 1 'Ausgewählt'.
FORMAT filter_$ (f1.0).
FILTER BY filter_$.
EXECUTE.
```

X

```
FILTER OFF.
USE ALL.
SELECT IF(filter_$).
EXECUTE.
```

…………………………………………………………………………………………………..

3. Order Variable I:

Before using the next syntax files, a new numeric variable named "Reihenfolge" allowing the data to be reordered without error, needs to be introduced in “Variablen Ansicht”. Next, open an EXCEL sheet and start an ascending enumeration from “1 – total number of cases in the SPSS data sheet”. Copy this series of numbers from the EXCEL sheet in the variable “Reihenfolge” on your SPSS data sheet. **This step is essential for the calculations of the foraging trip duration determination.**

If you are conducting a feeder experiment and are working with four scanners (two in front of the hive and two in front of the feeder chamber) different data sheets are better for the calculations of the durations of the different foraging trip phases. **Copy the actual SPSS datasheet four times (go to SPSS data sheet and do as follows: Daten -> Datenblatt kopieren, repeat this three times) in order to calculate “total foraging trip duration”, “duration of stay at the feeder”, “flight duration from hive to feeder and back again”, and “time interval spent inside the hive between foraging trips”. Save all four SPSS data sheets in appropriate fashion.**

…………………………………………………………………………………………………..

4. “Syntax for erasing unnecessary scanner data”:

for the calculation of the “total foraging trip duration” and “time interval spent inside the hive between foraging trips” only scanners from the hive are needed, for calculation of the “duration of stay at the feeder” only the feeder scanners are required, and for the calculation of the “flight duration from hive to feeder and back again” all four scanners are needed. Therefore the unnecessary scanner data from the feeder needs to be erased in the data sheets for the calculation of the “total foraging trip duration” and “time interval spent inside the hive between foraging trips” and the unnecessary scanner data from the hive needs to be erased in the data sheet for the calculation of the “duration of stay at the feeder”. No scanner data must

be erased from the data sheet for calculation of “flight duration from hive to feeder and back again”.

In the following example of the syntax, scanner 22350 and 22353 were positioned at the hive, while scanners 22352 and 27224 were positioned at the feeder. Each new SPSS data sheet automatically gets a certain DataSet number, visible in square brackets behind the filename shown in the upper left corner of the SPSS data sheet. In this case DataSet 18 was the data sheet for the calculation of the “duration of stay at the feeder” while DataSet 19 was the data sheet for the calculation of the “total foraging trip duration”. First part of the syntax erases all scanner data from the hive while the second part erases all data from the feeder. Beware to always have the right DataSet numbers inserted when using this syntax.

```
DATASET ACTIVATE DatenSet18.
FILTER OFF.
USE ALL.
SELECT IF (Scanner_ID = 22352 | Scanner_ID = 27224).
EXECUTE.

DATASET ACTIVATE DatenSet19.
FILTER OFF.
USE ALL.
SELECT IF (Scanner_ID ~= 22352  &  Scanner_ID ~= 27224).
EXECUTE.
```

……

5. "Syntax to calculate flight duration from hive to feeder and back again as well as total foraging trip duration":

The following syntax will calculate "flight duration from hive to feeder and back again" and the "total foraging trip duration". **Therefore only use on the two respective SPSS data sheets (see above).** It creates two new variables ("ftime", "used_ant"). "ftime" is the calculated time interval between defined antenna sequences and defined time periods between antenna registrations.
used_ant locks all cases of a defined antenna sequence so that they are locked and cannot be used again for the next antenna sequence.

Consecutive antenna sequences and time intervals for this syntax were:
Sequence 1: 1 0 0 1
(1 [time period ≤ 5seconds] 0 [time period ≥ 5sec] 0 [time period ≤ 5sec] 1)
Sequence 2a: 1 0 0
(1 [time period ≤ 5sec] 0 [time period ≥ 5sec] 0)
Sequence 2b: 0 0 1
(0 [time period ≤ 5sec] 0 [time period ≥ 5sec] 1)
Sequence 3: 1 0 1
1 [time period ≤ 5sec] 0 [time period ≥ 5sec] 1
Sequence 4: 1 0 1 1
(1 [time period ≤ 2sec] 0 [time period ≥ 2sec] 1 [time period ≥ 2sec] 1)

```
SORT CASES BY
 Reihenfolge(a).
COMPUTE
ftime=-1.
EXECUTE.

COMPUTE
used_ant=0.
EXECUTE.
```

1. antenna sequence: 1 0 0 1

```
IF ((((antenna=1 and lag(antenna)=0 and lag(antenna,2)=0 and lag(antenna,3)=1)
and ((lag(time)-time <=5) and (lag(time,3)-lag(time,2)<=5))
 and (tag=lag(tag,2) and date=lag(date,3))))) ftime = lag(time,2)-lag(time).
```

Used antennas from this sequence will be locked for sequence 2-4.

```
IF (ftime >-1) used_ant=4.
EXECUTE .

sort cases by
Reihenfolge (d) .

IF (lag(used_ant)=4) used_ant=3.
IF (lag(used_ant)=3) used_ant=2.
IF (lag(used_ant)=2) used_ant=1.
EXECUTE.

SORT CASES BY
 Reihenfolge(a).
```

2. antenna sequence either: 1 0 0

```
IF ((((antenna=1 and lag(antenna)=0 and lag(antenna,2)=0 ) and ((lag(time)-time
<=5) and (lag(time,2)-lag(time)>=5) and (used_ant=0)) and (tag=lag(tag,2) and
date=lag(date,2))))) ftime = lag(time,2)-lag(time).
```

or: 0 0 1

```
IF ((((antenna=0 and lag(antenna)=0 and lag(antenna,2)=1 ) and ((lag(time)-time
>=5) and (lag(time,2)-lag(time)<=5) and (used_ant=0)) and (tag=lag(tag,2) and
date=lag(date,2))))) ftime = lag(time)-time.
```

Used antennas from sequence 1 and 2 will be locked for sequences 3 and 4.

```
IF ((ftime >-1) and (used_ant=0)) used_ant=5.
EXECUTE .

sort cases by
Reihenfolge (d) .

IF (lag(used_ant)=5) used_ant=6.
IF (lag(used_ant)=6 and used_ant=0) used_ant=7.
EXECUTE.

IF ((antenna=0 and (lag(antenna)=1 or lag(antenna)=0)) and (used_ant=0 and
lag(used_ant)<>0)) used_ant=-1.
EXECUTE.
```

```
SORT CASES BY
Reihenfolge (a).
```

3. antenna sequence: 1 0 1

```
IF (((antenna=1 and lag(antenna)=0 and lag(antenna,2)=1) and (lag(time)-time < 5
and lag(time,2)-lag(time) >=5 and lag(time,3)-lag(time,2) > 5) and (used_ant=0 and
lag(used_ant,2)=0) and (tag=lag(tag,2) and date=lag(date,2))) and (lag(antenna,2)-
lag(antenna,3)<>1)) ftime = lag(time,2)-time.
```

used antennas from sequence 1,2, and 3 will be locked for sequence 4

```
IF ((ftime >-1) and (used_ant=0)) used_ant=8.
EXECUTE.

SORT CASES BY
Reihenfolge (d).


IF (lag(used_ant)=8 and used_ant=0) used_ant=9.
IF (lag(used_ant)=9 and used_ant=0) used_ant=10.
EXECUTE.

SORT CASES BY
Reihenfolge (d).
```

4. antenna sequence: 1 0 1 1

```
IF((((antenna=1 and lag(antenna)=0 and lag(antenna,2)=1 and lag(antenna,3)=1)
and (time-lag(time) <=2 and lag(time,2)-lag(time,3)>=2)) and (used_ant=0) and
(tag=lag(tag,2) and date=lag(date,2)))) ftime = time-lag(time,2).

IF ((ftime >-1) and (used_ant=0)) used_ant=11.
EXECUTE.

SORT CASES BY
Reihenfolge(a).

IF (lag(used_ant)=11) used_ant=12.
```

IF (lag(used_ant)=12) used_ant=13.
EXECUTE.

IF used_ant=1 ftime=-1.
EXECUTE.

……

6. “Syntax to calculate duration of stay inside the feeder compartment”:

The following syntax will calculate “duration of stay inside the feeder compartment”. **Therefore, only use it on the respective SPSS data sheet containing the data from the feeder scanners.** It creates two new variables (“ftime2”, “used_ant”).
“ftime2” is the calculated time interval between defined antenna sequences and defined time periods between antenna registrations.
used_ant locks all cases of a defined antenna sequence so that they are locked and cannot be used again for the next antenna sequence.

Consecutive antenna sequences and time intervals for this syntax were:
Sequence 1: 0 1 1 0
(0 [time period ≤ 5sec] 1 [time period ≥ 5sec] 1 [time period ≤ 5sec] 0)
Sequence 2a: 0 1 1
(0 [time period ≤ 5sec] 1 [time period ≥ 5sec] 1)
Sequence 2b: 1 1 0
(1 [time period ≤ 5sec] 1 [time period ≥ 5sec] 0)
Sequence 3: 0 1 0
(0 [time period ≤ 5sec] 1 [time period ≥ 5sec] 0)
Sequence 4: 1 0 1 1
(0 [time period ≤ 2sec] 1 [time period ≥ 2sec] 0 [time period ≥ 2sec] 0)

SORT CASES BY
Reihenfolge(a).
COMPUTE
ftime2=-1.
EXECUTE.

COMPUTE

```
used_ant_f=0.
EXECUTE.
```

1. antenna sequence: 0 1 1 0

```
IF ((((antenna=0 and lag(antenna)=1 and lag(antenna,2)=1 and lag(antenna,3)=0)
and ((lag(time)-time <=5) and (lag(time,3)-lag(time,2)<=5))  and (tag=lag(tag,2) and
date=lag(date,3))))) ftime2 = lag(time,2)-lag(time).

IF (ftime2 >-1) used_ant_f=44.
EXECUTE .

Sort cases by
Reihenfolge (d) .

IF (lag(used_ant_f)=44) used_ant_f=33.
IF (lag(used_ant_f)=33) used_ant_f=22.
IF (lag(used_ant_f)=22) used_ant_f=11.
EXECUTE.

SORT CASES BY
 Reihenfolge(a).
```

2. antenna sequence: either 0 1 1

```
IF ((((antenna=0 and lag(antenna)=1 and lag(antenna,2)=1 ) and ((lag(time)-time
<=5) and (lag(time,2)-lag(time)>=5) and (used_ant_f=0)) and (tag=lag(tag,2) and
date=lag(date,2))))) ftime2 = lag(time,2)-lag(time).
```

or: 1 1 0

```
IF ((((antenna=1 and lag(antenna)=1 and lag(antenna,2)=0 ) and ((lag(time)-time
>=5) and (lag(time,2)-lag(time)<=5) and (used_ant_f=0)) and (tag=lag(tag,2) and
date=lag(date,2))))) ftime2 = lag(time)-time.
IF ((ftime2 >-1) and (used_ant_f=0)) used_ant_f=55.
EXECUTE .

sort cases by
Reihenfolge (d) .

IF (lag(used_ant_f)=55) used_ant_f=66.
```

```
IF (lag(used_ant_f)=66 and used_ant_f=0) used_ant_f=77.
EXECUTE.

IF ((antenna=1 and (lag(antenna)=0 or lag(antenna)=1)) and (used_ant_f=0 and
lag(used_ant_f)<>0)) used_ant_f=-11
.
EXECUTE.

SORT CASES BY
Reihenfolge (a).
```

3. antenna sequence: 0 1 0

```
IF (((antenna=0 and lag(antenna)=1 and lag(antenna,2)=0) and (lag(time)-time < 5
and lag(time,2)-lag(time) >=5 and lag(time,3)-lag(time,2) > 5) and (used_ant_f=0 and
lag(used_ant_f,2)=0)  and (tag=lag(tag,2) and date=lag(date,2))) and lag(antenna,2)-
lag(antenna,3)<>1)) ftime2 = lag(time,2)-time.
IF ((ftime2 >-1) and (used_ant_f=0)) used_ant_f=88.
EXECUTE .

SORT CASES BY
Reihenfolge (d).

IF (lag(used_ant_f)=88 and used_ant_f=0) used_ant_f=99.
IF (lag(used_ant_f)=99 and used_ant_f=0) used_ant_f=100.
EXECUTE.

SORT CASES BY
Reihenfolge (d).
```

4. antenna sequence: 0 1 0 0

```
IF((((antenna=0 and lag(antenna)=1 and lag(antenna,2)=0 and lag(antenna,3)=0)
and (time-lag(time) <=5 and lag(time,2)-lag(time,3)>=5)) and (used_ant_f=0) and
(tag=lag(tag,2) and date=lag(date,2)))) ftime2 = lag(time)-lag(time,2).

IF ((ftime2 >-1) and (used_ant_f=0)) used_ant_f=111.
EXECUTE.
```

```
SORT CASES BY
Reihenfolge(a).

IF (lag(used_ant_f)=111 and used_ant_f=0) used_ant_f=122.
IF  (lag(used_ant_f)=122 and used_ant_f=0) used_ant_f=133.
EXECUTE.

IF used_ant_f=11 ftime2=-1.
EXECUTE.
```

…………………………………………………………………………………………………..

7. "Syntax to differentiate flights to the feeder from flights to the hive":

This syntax decides if a calculated flight duration was directed from hive to feeder or from feeder to hive.

Again the example uses scanner 22352 and 27224 as feeder scanners, and scanner 22350 and 22353 as hive scanners.

The syntax creates a new variable called "fdirection". If "fdirection" has a value of 1 the flight was headed from hive to feeder. If "fdirection" has a value of 2 the flight was headed from feeder to hive. **Only use this syntax on the SPSS data sheet containing both the data of the scanners in front of the hive and in front of the feeder.**

```
COMPUTE
fdirection=0.
EXECUTE.
SORT CASES by
Reihenfolge (a).
IF  (((scanner_ID=22352 | scanner_ID=27224) and ftime > 0) and
(lag(scanner_ID,2)<>22352) and (lag(scanner_ID,2)<>27224)) fdirection=2.

IF (((ftime > 0 and ((scanner_ID=22350) | (scanner_ID= 22353)))) and
(lag(scanner_ID,2)=22352 | lag(scanner_ID,2)=27224)) fdirection=1.
EXECUTE .
```

```
SORT CASES by
Reihenfolge (d).

IF (((scanner_ID=22352 | scanner_ID=27224) and ftime > 0 and used_ant=11) and
(lag(scanner_ID,2)<>22352) and (lag(scanner_ID,2)<>27224))  fdirection=1.

IF (ftime > 0 and used_ant=11  and (scanner_ID=22350 | scanner_ID= 22353) and
(lag(scanner_ID,2)=22352 | lag(scanner_ID,2)=27224)) fdirection=2.
EXECUTE.

SORT CASES by
Reihenfolge (a).
```

……………………………………………………………………………………………………..

8. “Syntax for calculation of the time period spent inside of the hive”:

This syntax calculates the time intervals spent inside of the hive between foraging flights. **Therefore, only use in on the respective SPSS data sheet.** It creates two new variables (“rtime_m” and “used_ant_m”). It is similar to the “Syntax to calculate duration of stay inside the feeder compartment”.

```
SORT CASES BY
 Reihenfolge(a).
COMPUTE
rtime_m=-1.
EXECUTE.

COMPUTE
used_ant_m=0.
EXECUTE.

IF ((((antenna=0 and lag(antenna)=1 and lag(antenna,2)=1 and lag(antenna,3)=0)
and ((lag(time)-time <=5) and (lag(time,3)-lag(time,2)<=5)) and (tag=lag(tag,2) and
date=lag(date,3))))) rtime_m = lag(time,2)-lag(time).

IF (rtime_m >-1) used_ant_m=44.
XX
```

```
EXECUTE .

sort cases by
Reihenfolge (d) .

IF (lag(used_ant_m)=44) used_ant_m=33.
IF (lag(used_ant_m)=33) used_ant_m=22.
IF (lag(used_ant_m)=22) used_ant_m=11.
EXECUTE.

SORT CASES BY
 Reihenfolge(a).

IF ((((antenna=0 and lag(antenna)=1 and lag(antenna,2)=1 ) and ((lag(time)-time
<=5) and (lag(time,2)-lag(time)>=5) and (used_ant_m=0)) and (tag=lag(tag,2) and
date=lag(date,2))))) rtime_m = lag(time,2)-lag(time).

IF ((((antenna=1 and lag(antenna)=1 and lag(antenna,2)=0 ) and ((lag(time)-time
>=5) and (lag(time,2)-lag(time)<=5) and (used_ant_m=0)) and (tag=lag(tag,2) and
date=lag(date,2))))) rtime_m = lag(time)-time.

IF ((rtime_m >-1) and (used_ant_m=0)) used_ant_m=55.
EXECUTE.

sort cases by
Reihenfolge (d) .

IF (lag(used_ant_m)=55) used_ant_m=66.
IF (lag(used_ant_m)=66 and used_ant_m=0) used_ant_m=77.
EXECUTE.

IF ((antenna=1 and (lag(antenna)=0 or lag(antenna)=1)) and (used_ant_m=0 and
lag(used_ant_m)<>0)) used_ant_m=-11.
EXECUTE.
```

```
SORT CASES BY
Reihenfolge (a).

IF (((antenna=0 and lag(antenna)=1 and lag(antenna,2)=0) and (lag(time)-time < 5
and lag(time,2)-lag(time) >=5 and lag(time,3)-lag(time,2) > 5) and  (used_ant_m=0
and lag(used_ant_m,2)=0)  and (tag=lag(tag,2) and date=lag(date,2))) and
lag(antenna,2)-lag(antenna,3)<>1)) rtime_m = lag(time,2)-time.

IF ((rtime_m >-1) and (used_ant_m=0)) used_ant_m=88.
EXECUTE.

SORT CASES BY
Reihenfolge (d).

IF (lag(used_ant_m)=88 and used_ant_m=0) used_ant_m=99.
IF (lag(used_ant_m)=99 and used_ant_m=0) used_ant_m=100.
EXECUTE.

SORT CASES BY
Reihenfolge (d).

IF((((antenna=0 and lag(antenna)=1 and lag(antenna,2)=0 and lag(antenna,3)=0)
and (time-lag(time) <=5 and lag(time,2)-lag(time,3)>=5)) and (used_ant_m=0) and
(tag=lag(tag,2) and date=lag(date,2)))) rtime_m = lag(time)-lag(time,2).

IF ((rtime_m >-1) and (used_ant_m=0)) used_ant_m=111.

EXECUTE.

SORT CASES BY
Reihenfolge(a).

IF (lag(used_ant_m)=111 and used_ant_m=0) used_ant_m=122.
IF  (lag(used_ant_m)=122 and used_ant_m=0) used_ant_m=133.
```

```
EXECUTE.

IF used_ant_m=11 rtime_m=-1.
EXECUTE.
```

....

9. The following syntax creates tables with number of determinable feeder compartment stays and the median duration of such a stay for every bee of each treatment group. In addition, a filter is applied that only searched for feeder stay durations longer than 30 seconds which was determined a minimum time period to forage on the sugar water. **Only use on the SPSS data sheet containing the data of the scanners positioned at the feeder (in this example DataSet29).**

```
DATASET ACTIVATE DatenSet29.
USE ALL.
COMPUTE filter_$=(ftime2 ~= lag(ftime2) & ftime2 > 30 & Behandlung > 0).
VARIABLE LABEL filter_$ 'ftime2 ~= lag(ftime2) & ftime2 > 30 & Behandlung > 0
(FILTER)'.
VALUE LABELS filter_$ 0 'Not Selected' 1 'Selected'.
FORMAT filter_$ (f1.0).
FILTER BY filter_$.
EXECUTE.

CTABLES
  /VLABELS VARIABLES=Behandlung Number ftime2 Date DISPLAY=LABEL
  /TABLE Behandlung [C] > Number > ftime2 [COUNT F40.0, MEDIAN] BY Date
  /CATEGORIES VARIABLES=Behandlung Number Date ORDER=A KEY=VALUE
EMPTY=EXCLUDE.
```

....

10. The following syntax creates tables with number of determinable foraging trips and the median duration of such a trip for every bee of each treatment group. In addition, a filter is applied that only searched for foraging trip durations longer than 30 seconds which was determined a minimum foraging period. **Only use on the SPSS data sheet containing the data of the scanners positioned at the hive (in this example DataSet1).**

```
DATASET ACTIVATE DatenSet1.
USE ALL.
COMPUTE filter_$=(ftime ~= lag(ftime) & ftime > 30 & Behandlung > 0).
VARIABLE LABEL filter_$ 'ftime ~= lag(ftime) & ftime > 30 & Behandlung > 0
(FILTER)'.
VALUE LABELS filter_$ 0 'Not Selected' 1 'Selected'.
FORMAT filter_$ (f1.0).
FILTER BY filter_$.
EXECUTE.

CTABLES
  /VLABELS VARIABLES=Behandlung Number ftime Date DISPLAY=LABEL
  /TABLE Behandlung [C] > Number > ftime [COUNT F40.0, MEDIAN] BY Date
  /CATEGORIES VARIABLES=Behandlung Number Date ORDER=A KEY=VALUE
EMPTY=EXCLUDE.
```

………………………………………………………………………………………………

11. The following syntax creates tables with number of determinable foraging flights to the feeder and back to the hive and the median duration of flights for every bee of each treatment group. In addition, a filter is applied that only searched for flight durations longer than 2 seconds which was determined a minimum flight duration. **Only use on the SPSS data sheet containing the data of the scanners positioned at the hive and at the feeder (in this example DataSet31).**

```
DATASET ACTIVATE DatenSet31.
USE ALL.
COMPUTE filter_$=(ftime ~= lag(ftime) & ftime > 2 & Behandlung > 0 & fdirection >
0).
```

```
VARIABLE LABEL filter_$ 'ftime ~= lag(ftime) & ftime > 2 & Behandlung > 0 &
fdirection > 0 '+
   '(FILTER)'.
VALUE LABELS filter_$ 0 'Not Selected' 1 'Selected'.
FORMAT filter_$ (f1.0).
FILTER BY filter_$.
EXECUTE.

CTABLES
  /VLABELS VARIABLES=Behandlung Number ftime Date fdirection
DISPLAY=LABEL
  /TABLE Behandlung [C] > Number > ftime [COUNT F40.0, MEDIAN] BY Date >
fdirection [C]
  /CATEGORIES VARIABLES=Behandlung Number Date fdirection ORDER=A
KEY=VALUE EMPTY=EXCLUDE.
```

....

12. The following syntax creates tables with number of determinable time intervals inside of the hive and the median duration of these in hive stays for every bee of each treatment group. In addition, a filter is applied that only searched for in hive stays longer than 30 seconds which was determined a minimum time period spent inside the hive between foraging trips. **Only use on the separate SPSS data sheet containing the data of the scanners positioned at the hive (in this example DataSet3).**

```
DATASET ACTIVATE DatenSet3.
USE ALL.
COMPUTE filter_$=(rtime_m ~= lag(rtime_m) & rtime_m > 30 & Behandlung > 0).
VARIABLE LABEL filter_$ 'rtime_m ~= lag(rtime_m) & rtime_m > 30 & Behandlung >
0 (FILTER)'.
VALUE LABELS filter_$ 0 'Not Selected' 1 'Selected'.
FORMAT filter_$ (f1.0).
FILTER BY filter_$.
EXECUTE.
```

```
CTABLES
  /VLABELS VARIABLES=Behandlung Number rtime_m Date DISPLAY=LABEL
  /TABLE Behandlung [C] > Number > rtime_m [COUNT F40.0, MEDIAN] BY Date
  /CATEGORIES VARIABLES=Behandlung Number Date ORDER=A KEY=VALUE
EMPTY=EXCLUDE.
```

……………………………………………………………………………………………………

13. The following preparations are necessary to identify the first and second stay inside of the hive between foraging trips after treatment. All determined stays inside of the hive that are shorter than 31 seconds are erased from the SPSS data sheet (only use on the respective data sheet, e.g. **“time interval spent inside the hive between foraging trips”)** and will sort the data in a descending fashion by date, number, and time.

```
DATASET ACTIVATE DatenSet3.
USE ALL.
FILTER OFF.
USE ALL.
SELECT IF (rtime_m > 30).
EXECUTE.

SORT CASES by
Date(D) Number(D) Time(D).
```

……………………………………………………………………………………………………

A new Order-Variable needs to be created before continuing (in this example “Reihenfolge2”, see also 2. “Order Variable I”)

14. “Syntax determining the first and second in hive stay”:

The following syntax is searching the data sheet for the first and second stay of each bee inside of the hive after treatment. The variable “first_record” is created. If the value of first record is “1” this labels the first in hive stay, if the value is “2” it labels the second in hive stay.

```
Reihenfolge2 (d).
```

```
COMPUTE
first_record=0.
EXECUTE.

IF Number <> lag(number) and rtime_m > 30  first_record=1.
IF lag(first_record)=1 & Number = lag(number) & rtime_m > 30 first_record=2.

EXECUTE.
```

After execution of the previous syntax the values “1” and “2” have to be inserted by hand into the first two cases of the variable “first_record”.

....

15. The following syntax creates tables with the duration of first and second in hive stay for every bee of each treatment group. **Only use on the respective SPSS data sheet (e.g. “time interval spent inside the hive between foraging trips”).**

```
USE ALL.
COMPUTE filter_$=(first_record > 0).
VARIABLE LABEL filter_$ 'first_record > 0 (FILTER)'.
VALUE LABELS filter_$ 0 'Not Selected' 1 'Selected'.
FORMAT filter_$ (f1.0).
FILTER BY filter_$.
EXECUTE.

CTABLES
  /VLABELS VARIABLES=Behandlung Number rtime_m Date first_record
DISPLAY=LABEL
  /TABLE Behandlung [C] > Number [C] > rtime_m [S][MEDIAN] BY Date [C] >
first_record [C]
  /CATEGORIES VARIABLES=Behandlung Number Date first_record ORDER=A
KEY=VALUE EMPTY=EXCLUDE.
```

....

For statistical analysis, the data from the tables (step 9-12, 15) need to be transferred into a new SPSS data sheet by either “copy and paste” from table to SPSS data sheet or by exporting the table to a word file followed by “copy and paste” to the nex SPSS data sheet.

The variables are chosen as follows (Variablen Ansicht):

Name	Typ	Spaltenf...	Dezimal...	Variablenlabel	Wertelabels
Date	Datum	10	0		{20.07.2010, b.t.}...
Treatment	Numerisch	8	0		{1, Control}...
Number	Numerisch	8	0		Keine
Count_Feeder	Numerisch	8	0		Keine
Median_Feeder_ftime	Numerisch	8	2	Time at the feeder [sec]	Keine
Count_Minis	Numerisch	8	0		Keine
Median_Minis_ftime	Numerisch	8	2	Foraging trip [sec]	Keine
Count_to	Numerisch	8	0	N= Feedervisits / bee	Keine
Median_to_ftime	Numerisch	8	2	Flight time to the feeder [sec]	Keine
Count_back	Numerisch	8	0		Keine
Median_back_ftime	Numerisch	8	2	Flight time to the hive [sec]	Keine
Count_inter_times_mini	Numerisch	8	0		Keine
Median_inter_mini	Numerisch	8	2	Period between two foraging trips [sec]	Keine
first_interval	Numerisch	8	0	1. Interval inside the hive after treatment...	Keine
sec_interval	Numerisch	8	0	2. Interval inside the hive after treatment...	Keine

Example of a data sheet (Clothianidin Experiment 20.07.2010):

Date	Treatment	Number	Count_Feeder	Median_Feeder_ftime	Count_Minis	Median_Minis_ftime	Count_to	Median_to_fti...	Count_back	Median_back_ftime	Count_inter_times_mini	Median_inter_mini	first_interval	sec_interval
20.07.2010	1	28549	25	93,00	23	130,00	24	11,00	23	13,00	26	111,00	645	145
20.07.2010	1	28566	9	110,00	5	325,00	4	121,00	6	23,50	6	968,00	1473	685
20.07.2010	1	28582	33	74,00	33	103,00	30	8,00	32	10,50	34	102,50	811	118
20.07.2010	1	28621	31	66,00	29	103,00	27	8,00	26	12,50	29	127,00	484	280
20.07.2010	1	28672	26	77,50	27	114,00	27	12,00	26	10,50	27	124,00	849	151
20.07.2010	1	44820	25	90,00	27	119,00	23	12,00	25	14,00	28	115,00	919	132
20.07.2010	1	44823	19	115,00	20	144,00	19	12,00	19	15,00	22	130,00	1535	70
20.07.2010	1	44876	32	71,00	30	99,50	31	10,00	30	10,50	34	114,50	358	114
20.07.2010	1	44926	26	94,00	25	134,00	25	15,00	24	10,50	26	130,50	1018	108
20.07.2010	1	61319	18	105,00	18	145,00	18	16,00	16	13,00	18	157,50	839	125
20.07.2010	1	61329	13	166,00	10	265,00	10	15,00	9	16,00	10	407,00	812	578
20.07.2010	2	28602	20	104,00	19	134,00	19	13,00	19	10,00	20	166,50	1193	85
20.07.2010	2	28608	9	137,00	10	190,00	10	16,00	10	13,50	10	460,50	1160	651
20.07.2010	2	28613	29	77,00	28	103,50	28	12,00	26	11,50	29	126,00	1345	104
20.07.2010	2	44807	33	76,00	32	104,50	32	8,50	30	14,00	34	99,00	435	88
20.07.2010	2	44812	35	70,00	39	89,00	36	8,00	35	11,00	41	85,00	896	113
20.07.2010	2	44827	22	147,00	21	191,00	21	13,00	20	11,00	22	90,00	1310	104
20.07.2010	2	44891	30	89,50	28	122,00	25	7,00	28	12,00	28	111,50	745	141
20.07.2010	2	44900	26	64,00	24	111,00	23	14,00	23	14,00	26	139,00	1170	164
20.07.2010	2	61313	32	72,50	31	101,00	30	13,00	32	13,50	38	100,50	636	94
20.07.2010	2	61367	17	88,00	19	127,00	18	16,00	18	13,50	21	195,00	1079	42
20.07.2010	2	61393	29	64,00	29	105,00	28	11,00	23	19,00	25	127,00	725	139
20.07.2010	3	28554	6	169,00	8	200,50	7	15,00	6	14,00	9	552,00	926	663
20.07.2010	3	28578	18	77,50	19	118,00	16	14,00	15	10,00	19	265,00	862	200
20.07.2010	3	28601	25	91,00	21	138,00	22	8,00	19	9,00	22	116,50	1182	134
20.07.2010	3	28611	19	127,00	18	212,00	16	13,50	15	16,00	17	148,00	899	899
20.07.2010	3	28629	21	144,00	19	182,00	18	16,00	16	13,50	19	142,00	934	204
20.07.2010	3	28630	19	84,00	17	119,00	17	11,00	18	15,50	17	177,00	267	303
20.07.2010	3	28632	32	80,00	32	109,00	33	8,00	32	12,00	36	83,50	692	79
20.07.2010	3	28637	31	68,00	33	96,00	33	9,00	30	9,00	34	113,50	565	114
20.07.2010	3	44815	26	88,50	18	124,00	21	9,00	21	18,00	21	122,00	1509	145
20.07.2010	3	44884	30	71,50	30	102,00	30	10,50	29	9,00	30	95,00	1245	93
20.07.2010	3	44923	22	93,50	21	122,00	21	9,00	21	12,00	22	146,50	1160	135
20.07.2010	3	61407	11	82,00	22	126,50	16	15,00	13	14,00	25	126,00	993	345
20.07.2010	4	28576	23	81,00	25	113,00	23	18,00	22	10,50	26	167,50	803	170

....

The following syntax executes a "oneway ANOVA" to analyze the influence of the different treatments on the foraging activity (number of feeder visits in three hour observation period). The different groups are further compared by a Bonferroni Post-hoc-Test.

Count_to= Number of flights from the hive to the feeder (foraging trips)

```
DATASET ACTIVATE DatenSet6.
SORT CASES  BY Datum.
SPLIT FILE SEPARATE BY Datum.
ONEWAY Count_to
BY Treatment
  /MISSING ANALYSIS
  /POSTHOC=BONFERRONI ALPHA(0.05).
```

…………………………………………………………………………………………………..

For statistical analysis of the different phases of a foraging trip syntaxes for non-parametrical Kruskal-Wallis- and Mann-Whitney-U-Tests were performed. The dependent variable was the different treatment. Treatment value for the different insecticide doses was as follows:

Imidacloprid:	Clothianidin	Coumaphos
1= Control	1= Control	1= Control
2= 0.15ng	2= 0.05ng	2= 1µg
3= 1.5ng	3= 0.5ng	3= 2µg
4= 3ng	4= 1ng	4= 5µg
5= 6ng	5= 2ng	

```
SORT CASES  BY Datum.
SPLIT FILE SEPARATE BY Datum.

DATASET ACTIVATE DatenSet1.
NPAR TESTS
```

Median_Minis_ftime= median foraging trip duration;

Median_to_ftime= median flight duration to the feeder;

Median_Feeder_ftime= median duration of stay inside the feeder compartment;

Median_back_ftime= median flight duration back to the hive;

Median_inter_mini= median time interval inside of the hive;

first_interval= first time interval inside of the hive after treatment;

sec_interval= second time interval inside of the hive after treatment.

```
  /M-W= Median_Minis_ftime Median_to_ftime Median_Feeder_ftime
Median_back_ftime Median_inter_mini first_interval sec_interval
    BY Treatment(1 2)
  /MISSING ANALYSIS.

NPAR TESTS
  /M-W= Median_Minis_ftime Median_to_ftime Median_Feeder_ftime
Median_back_ftime Median_inter_mini first_interval sec_interval
    BY Treatment(1 3)
  /MISSING ANALYSIS.

NPAR TESTS
  /M-W= Median_Minis_ftime Median_to_ftime Median_Feeder_ftime
Median_back_ftime Median_inter_mini first_interval sec_interval
    BY Treatment(1 4)
  /MISSING ANALYSIS.

NPAR TESTS
  /M-W= Median_Minis_ftime Median_to_ftime Median_Feeder_ftime
Median_back_ftime Median_inter_mini first_interval sec_interval
    BY Treatment(1 5)
  /MISSING ANALYSIS.
```

XV. Ehrenwörtliche Erklärung

Christof Schneider

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Ehrenwörtliche Erklärung zu meiner Dissertation
mit dem Titel:

„Detecting the influence of different potential stress factors on the behavior of the honeybee *Apis mellifera* using Radiofrequency Identification (RFID)“

Sehr geehrte Damen und Herren,

hiermit erkläre ich, dass ich die beigefügte Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel genutzt habe. Alle wörtlich oder inhaltlich übernommenen Stellen habe ich als solche gekennzeichnet.

Ich versichere außerdem, dass ich die beigefügte Dissertation nur in diesem und keinem anderen Promotionsverfahren eingereicht habe und, dass diesem Promotionsverfahren keine endgültig gescheiterten Promotionsverfahren vorausgegangen sind.

_________________________ ____________________________________

Ort, Datum Unterschrift