Review paper

PROSPECTS AND VALIDITY OF LABORATORY CAGE TESTS CONDUCTED IN HONEYBEE RESEARCH PART TWO: NEW POSSIBILITIES FOR USE OF LABORATORY CAGE TESTS IN RESPONSE TO CHALLENGES REVEALED AT THE TURN OF THE 20TH AND 21ST CENTURIES

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Abstract

Nowadays, cell cultures are a standard tool in animal biotechnology, but the problem with honeybees is the constant lack of appropriate cell lines to be used in in vitro research. Until the imperfections of bee tissue cultures are resolved, researchers have to conduct experiments on bees in laboratory cage tests (LCTs).

At the turn of the 21st century many new hazards for beekeeping appeared. An early recognized problem was the Colony Collapse Disorder and Honey Bee Depopulation Syndrome, which were associated with the harmfulness of pesticides and strictly linked with a decline in bee immunity. Such problems in LCTs were attempted to be resolved through research on the interactions between biostimulators and antiparasitic drugs. LCTs allow the relationship between the dose of a specific factor and its impact to be determined, which can be used in the establishment of reference values. Furthermore, LCTs may be a useful tool in understanding the function and role of bee gut flora.

Using the honeybee as an animal model is possible thanks to knowledge of the honeybee genome and bee biology and the similarity between some physiological and biochemical processes and those occurring in humans. So far, LCTs have been used to understand better human aging, learning and gene expression regulating. This is facilitated by the advanced development of medicine and molecular genetics, and in the future the use of honeybees may become a standard in biochemical or gerontological research.

Keywords: animal model, *Apis mellifera*, honeybee, laboratory cage tests

Laboratory cage tests as an alternative of cell line culturing in honeybee research

Cell cultures have become a standard tool used in biotechnology, biopharmacy, and toxicology. Cell lines of human or animal cells cultured and multiplied in appropriate and often specific conditions, allow the influence of various substances, including drugs, on their vitality and functions to be studied (Stasiak & Sznitowska, 2010). Stokłosowa (2004) studied the use of cell cultures to predict the toxic properties of various chemical substances. As with animals or humans, there are procedures for breeding the cell cultures of bees (Hunter, 2010; Goblirsch et al., 2013) and other insects (Lyn, 2002). However, the honeybees still lack clean, disease-free and stable cell lines which could be used in *in vitro* studies (Genersch et al., 2013). The AmE-711 line derived in 2011 is characterized by a long adaptation time, but research on the possibilities and factors of its differentiation into specific tissues is required, which would allow research on more specific aspects (Goblirsch et al., 2013; Goblirsch, 2017). Until the imperfec-

tions of bee tissue cultures are not resolved, the researchers must conduct experiments directly on bees in laboratory cage tests (LCT). These tests are useful in the research of bee diseases, especially viruses and nosemosis (Goblirsch, 2017), because they give a chance to learn how pathogens affect a single bee organism and thus to predict their impact on the colony.

Honeybee as an animal model organism

The honeybee has high phenotypic plasticity, highly similar physiological and biochemical processes to those in mammals including humans (Lockett, Helliwell, & Maleszka, 2010; Strachecka et al., 2012a; Strachecka et al., 2015) and achievements resulting from social life (Keller & Jemielity, 2006) and so can be considered as a model organism in studies on longevity (Keller & Jemielity, 2006; Eyer et al., 2017), epigenetic changes (Kucharski et al., 2007; Lockett, Wilkes, & Maleszka, 2010; Paleolog et al., 2011; Strachecka et al., 2012a) and selected factors' influence on the functioning of the organism (Si et al., 2005; Strachecka et al., 2012d). Given the high similarity to humans, bees can be used to study the action of selected substances in the organism.

For example amphotericin B has been found to be deposited in internal organs in bees as well as humans (Gagoś et al., 2011; Strachecka et al., 2012d). Additionally, this antibiotic in bees increases the concentration of proteins on the surface of their bodies, reduces the level of total DNA methylation affecting the equilibrium of the demethylation/methylation epigenetic mechanisms, and shortens the lifespan of bees. Similar side effects can be seen in other bees antibiotics (Howis et al., 2012; Strachecka et al., 2012d). Furthermore, the multifactorial effect of curcumin is an activator of numerous biochemical processes in bees (Strachecka et al., 2015) and mammals (Trujillo et al., 2013).

An equally important trend, honeybees are used as a model organism in research on aging. This process in the honeybee varies depending on the caste. Despite the similar genotype, the queen lives much longer than the workers, a phenomenon called phenotypic plasticity. Such differences in life expectancy are the result of DNA methylation, which involves the attachment of a methyl group (-CH^3) to the CpG islands, i.e. DNA regions with high dinucleotide frequency. A set of either methylation points or enzymes necessary for CpG methylation that the honey bee possesses predisposes it as a model for epigenetic research among other insects (Strachecka et al., 2012a). Lockett, Helliwell & Maleszka (2010) found that methylation occurred analogously in specific CpG in the honeybee in response to training as in rats. The difference in the gene methylation level is already evident during the larval stage and results from the quality of larval feeding leading to phenotypic plasticity (Münch, et al., 2008; Strachecka et al., 2012a). This illustrates the importance of the composition of the diet in gene expression regulation and, consequently, longevity. In many animals, life expectancy is also prolonged by insulin/insulin-like growth factor (IIS), and this is the case with bees where the IIS level depends on workers' caste and age (Corona et al., 2007; Yan et al., 2015). In addition, the occurrence of winter workers, lifespan up to six months, and summer bees, lifespan about four weeks, is regulated by biochemical and epigenetic mechanisms (Strachecka et al., 2012d), whose aspects outlined here are highly interrelated and complex and, at the same time, modified by environmental factors. Hence, the level of influence of a single factor or substance on these aspects can be captured and objectively assessed in LCTs.

Honeybees and humans correspond in processes related to learning and remembering mechanisms. Memory formatting in bees is determined by neurotransmitters and mediators (Si et al., 2005), similar to those present in humans (Kucharski et al., 2007). The high socialization of bees' behavior allows them to be considered as one of the best models in the study of learning and remembering (Menzel & Giurfa, 2001; Strachecka et al., 2012a). Long-term memory, learning, and remembering are processes guided by DNA methylation, and the bee learning model itself is similar to

that of vertebrates (Dyer, 2005; Biergans et al., 2012). In bees, memory and learning tests are conducted at least with the help of LCTs, based on the necessity for the bee colony to find and acquire food (Decourtye et al., 2004; Jones et al., 2005). LCTs allow comfortable processing and repeating learning processes and facilitate easy identification of single bees and individual handling thereof (Decourtye et al., 2003; Si et al., 2005; Agarwal et al., 2011).

Individual immunity and bee diseases

The first line of defense against pathogens is the surface of the bee's body, including the layer of biologically active proteins on the surface of the cuticle. These proteins have e.g. the properties of proteases (Evans et al., 2006; Evans & Spivak, 2010; Strachecka et al., 2010; Strachecka et al., 2018), which protect insects against infections and help maintain body homeostasis (Brownlees & Williams, 1993; Gorman & Paskewitz, 2001).

Bees can be infected with pathogens when taking or sharing infected food (Cremer & Sixt, 2009; Hamilton et al., 2010). On the other hand, drugs or agents can be inserted into the alimentary tract of stimulating individual bee immunity in conjunction with food. LCTs are convenient assessing the impact of such substances on bees and their parasites (Costa et al., 2010; Borsuk et al., 2013; Strachecka et al., 2014; Strachecka et al., 2015). Studies of nosemosis are a good way to use LCTs, as this fungal disease commonly causes significant economic losses with its high infectivity. In this case, the use of LCTs allows the precise distribution of the pathogen in the whole group (Fries et al., 2013), the individual feeding of infected bees (Pettis et al., 2012; Borsuk et al., 2018) and identification of the effect of the tested factors during disease development (Costa et al., 2010). LCTs prevent the spread of nosemosis and the robbing and straying of bees, which occur when such tests are conducted in apiary conditions.

However, the biggest hazard for the present beekeeping is varrosis, a disease caused by the parasitic mite *Varroa destructor* (Rosenkranz et al., 2010). The widespread presence of the parasite and large economic losses resulting from the paralysis of colonies make chemotherapy the basis for limiting invasion. Synthetic substances with acaricidal agents (Bąk et al., 2010) and organic acids (Imdorf et al., 2003; Strachecka et al., 2013) are used predominantly. Although commercial preparations containing these substances are referred to as medicines, their effects are not neutral for bees. This is why LCTs have also been used to assess how acaricides impact the individual resistance in workers. Strachecka et al. (2012b, 2012c) showed that amitraz and oxalic acid in apiary conditions reduce or/and cause a loss in activity of the proteolytic system in bees. Moreover, the activity of the cuticle proteolytic system has been found to respond to antibiotic therapy in similar way as to amitraz, the active substance of many commercial products against *Varroa destructor* (Howis et al., 2012; Strachecka et al., 2012b, 2012d). Johnson et al. (2013) conducted a series of tests aimed at capturing the relationship between such acaricides as amitraz, tau-fluvalinate, thymol, kumafos and fungicides and between these acaricides and antimicrobials to determine the lethal effects of individual substances on bees and interactions between them. Consequently, the interactions between different chemicals in bees were demonstrated to be as complex as drug interactions in mammals (Johnson et al., 2013). In conclusion, substances that are commonly used in apiaries as acaricides cause a number of adverse changes in bee resistance. Besides the assessment of their effectiveness, the use of LCTs also allows assessment of side effects of chemotherapy at the level of an individual bee.

The downsides of the nutritional base, mainly pollen deficiencies and difficulty in controlling varrosis and nosemosis, result in a decreased individual immunity of bees and, consequently, a decline in the condition of colonies. This situation forces beekeepers to use biostimulants and supplements to improve the health of bee colonies (Szymaś & Przybył, 2007; Strachecka et al., 2015). The preliminary study of the impact of such substances on bees, and thus the assessment of their suitability in LCTs, is faster and cheaper than in bee colonies due to

low labor intensity and use of small numbers of bees and small amount evaluated substance. This direction of research includes the assessment of the impact of nanosilver on *Nosema* spp. The supplementation of sugar syrup with nanosilver at a 25-ppm concentration may limit the invasion of *Nosema* spp. (Borsuk et al., 2013). Ptaszyńska et al. (2018) obtained promising results in the fight against *Nosema* spp. by using porphyrin for disease control. When evaluated in laboratory conditions, caffeine also inhibited the development of *Nosema* spp. The development chain of *Nosema* spp. is inhibited probably due to the high activity of the proteolytic system, in particular protease inhibitors, or reduced accessibility of amino acids necessary for the production of parasite proteins (Mello & Silva-Filho, 2002; Strachecka et al., 2014). Moreover, 5µg/ml of the caffeine addition slows the epigenetic processes of DNA methylation associated with the aging process, significantly extending the life of bees. Therefore that the effect of caffeine can be assumed to be bidirectional. It inhibits the development of *Nosema* spp. on the one hand and intensifies or regulates biochemical processes on the other (Strachecka et al., 2014). Curcumin turned out to be an unexpectedly effective natural biostimulator. Already at a dose of 3 µg/ml (an equivalent dose for humans), it improved the condition and vitality of bees, which in turn had a longer lifespan (Strachecka et al., 2015).

New threats from pesticides

Despite the strict pesticide authorization procedures, bee poisonings continue to be a constant problem (Blacquière et al., 2012). At the turn of the 20th and 21st centuries, the harmfulness of insecticides prompted researchers to check their relationship with two alarming occurrences The Honeybee Depopulation Syndrome and Bee Colony Collapse Disorder. Their causes were linked with e.g. substances from the neonicotinoid group (Godfray et al., 2015; Woodcock et al., 2016), which, especially the widely used imidacloprid, have potent neurotoxic effects. LCTs are often the basis to discover the sources and mechanisms of the harmful effects of chemical substances on bees, which are unknown at the time of licensing permit procedures, which was the case with imidacloprid. A rapid appearance of neurotoxicity symptoms, manifested by problems with motor coordination and convulsions was found in a LCT (Suchail et al., 2001). These reactions are considered as the most probable cause of death of some workers before they return to the nest from contaminated plantations (Decourtye et al., 2003, 2004). The described symptoms gradually disappear within a few hours. Workers begin to show inactivity and a lack of reaction to external stimuli, which is part of the chronic toxicity of insecticides (Suchail et al., 2001; Medrzycki et al., 2013). The scale of pesticides' toxic effects on bees necessitates continuous improvement of methodologies for the assessment of their toxicity where the LCT is an indispensable element of related procedures. Decourtye et al. (2004) discovered that some neonicotinoid compounds cause learning disabilities only in cage tests, with no analogy in the semi-field test and showed how environmental influences on the studied factors are significant in bee research and emphasizes the need for verification of LCT results in apiary conditions.

Mechanisms of function and the role of bee digestive tract flora

The flora of the digestive tract is an important element that influences the basic functions of organisms (Engel, Martinson, & Moran, 2012; Jones et al., 2018). However, it is not sufficiently known how it does so on the health of individual bees and the biology of bee colonies (Jones et al., 2018), so LTCs could be a useful tool in the exploration of this issue. The flora of the bee alimentary canal, including fructophilic lactic acid bacteria (FLAB), is a derivative of the environment (Endo et al., 2009). Bees acquire non-endogenous microorganisms mainly while collecting fructose-rich nectar (McFredrick et al., 2012; Endo & Salmien, 2013). Fructophilic bacteria accompanying bees are chosen by some researchers for their probiotic applications (Endo & Salmien, 2013) and as paratransgenic agents in the fight against European foulbrood (Rangberg et al., 2015; Erban et al., 2017). This

is connected with FLAB commonly inhabiting the digestive tract, which influences the direct inhibition of the growth of other microorganisms by acidification of the intestinal environment and production of such antimicrobial compounds as hydrogen peroxide, organic acids and antimicrobial peptides (Audisio et al., 2011). FLAB participate in the constant stimulation of the intestinal immune system (Engel, Martinson, & Moran, 2012). In addition, it has been shown that the entire gastrointestinal microflora in bees has a significant impact on the production of pheromones, degradation of pesticides and synthesis of vitamins (Engel, Martinson, & Moran, 2012). Since the microflora of the gastrointestinal tract of 6-day-old workers is fully developed, compared to freshly emerged bees (Powell et al., 2014), it is reasonable to use LCTs based on freshly emerged sterile workers (Vojvodic, Rehan, & Anderson, 2013) free of FLAB. Such a procedure yields a 100% response of the bee organism.

Conclusions and prospects for LCTs

Currently in the 21st century, contemporary challenges for beekeeping as well as humanity have created new opportunities for LCTs, which facilitate a more complete use of bees as model organisms, which, among other things, gives the opportunity to elucidate important processes for humans. LCTs allow a better understanding of bees including their genome, social behavior, mechanisms of the nervous system function and biochemical processes showing considerable analogies to those in mammals and even in humans and with the use of honeybees may become a standard in medical research in the near future. Their low costs, easy handling, small groups of individuals caged in controlled conditions, and the possibility of individual treatment of a single bee makes them a promising tool for honeybee research.

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