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REVIEW ARTICLE

Standard methods for artificial rearing of *Apis mellifera* larvae

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Summary

Originally, a method to rear worker honey bee larvae *in vitro* was introduced into the field of bee biology to analyse honey bee physiology and caste development. Recently, it has become an increasingly important method in bee pathology and toxicology. The *in vitro* method of rearing larvae is complex and can be developed as an art by itself, especially if the aim is to obtain queens or worker bees which, for example, can be re-introduced into the colony as able members. However, a more pragmatic approach to *in vitro* rearing of larvae is also possible and justified if the aim is to focus on certain pathogens or compounds to be tested. It is up to the researcher(s) to decide on the appropriate experimental establishment and design. This paper will help with this decision and provide guidelines on how to adjust the method of *in vitro* rearing according to the specific needs of the scientific project.

Métodos estándar para la cría *in vitro* de larvas de *Apis mellifera*

Resumen

Originalmente, el método para la cría *in vitro* de larvas de obreras de abejas melíferas se introdujo en el campo de la biología de las abejas para analizar la fisiología y el desarrollo de las castas. Recientemente, se ha convertido en un método cada vez más importante para la patología y la toxicología de la abeja. El método de cría *in vitro* de larvas es complejo y constituye un arte en sí mismo, especialmente si el objetivo es obtener reinas o abejas obreras que, por ejemplo, puedan ser re-introducidas en la colonia como miembros activos. Sin embargo, un enfoque más pragmático de la cría de larvas *in vitro* también es posible y justificado si el objetivo es centrarse en ensayos con ciertos patógenos o compuestos. Corresponde al investigador (es) decidir sobre el adecuado establecimiento experimental y el diseño. Este artículo ayudará con esta decisión y proporcionará directrices sobre cómo ajustar el método de cría *in vitro* en función de las necesidades específicas del proyecto científico.

人工饲养西方蜜蜂幼虫的标准方法

人工饲养工蜂幼虫的方法最早始于蜜蜂生物学研究，用来分析蜜蜂的生理机能和级型发育。近来，其在蜜蜂病理学和毒理学领域的应用也日趋重要。人工饲养幼虫方法十分复杂但也极富技巧，特别是当培养目的是获得可以重新介绍入蜂群中的蜂王或工蜂，并还可成为群体的有效成员时将更加困难。 目前一个经过改进并更为实用的饲养技术方案已经形成，以此技术方案为基础，略加调整就可成为用于某单一病原体或混合病原体

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测试实验所需的饲养方案。由此类推，研究者可根据自己的研究目的，以本方案为基础，经再加工就可设计或调整出可满足自己实验需要的饲养方案。本文针对各类科研项目的需求，就如何调整人工饲养蜜蜂幼虫方案给出了指导方针。

Keywords: honey bee, *Apis mellifera,* larval rearing, standardization, risk assessment, *BEEBOOK*, COLOSS

1. Introduction

Honey bees are important pollinators and are responsible for much of the world's agricultural production and the conservation of biodiversity (Klein *et al.*, 2007; Gallai *et al.*, 2009). In many regions of the world, the number of honey bee colonies is declining, thus possibly endangering pollination (Aizen *et al.*, 2009; VanEngelsdorp and Meixner, 2010; van der Zee *et al.*, 2012). Parasites, pathogens and pesticides are three of the major threats to honey bees and are believed to be partially responsible for the abovementioned declines (Neumann and Carreck, 2010). Therefore, the effects of these handicaps and the combination of two or more sublethal effects are extensively investigated (Mullin *et al.,* 2010; Moritz *et al.*, 2010; Genersch *et al.*, 2010). An important tool for this research is the rearing of honey bee larvae *in vitro* (i.e. in the laboratory and in the absence of nurse bees) because it allows more controllable conditions compared to *in vivo* (i.e. in the hive by nurse bees). However, artificial larval rearing can also be regarded as an *in vivo* method conducted in an *in vitro* system. In particular, the testing of the toxicity of plant protection products on brood can be conducted in a reproducible and standardized way only in the laboratory, because a defined uptake of food containing the testing compound is not feasible using in-hive methods (Wittmann and Engels, 1981; Oomen *et al.*, 1992; Schuur *et al.*, 2003; Becker *et al.,* 2009; Aupinel *et al.,* 2007a).

The first attempt at mass application and standardization of *in vitro* rearing of honey bee larvae and testing plant protection products was a ring test, in which seven different laboratories assessed the LD_{50} for dimethoate 48 hours after acute larval exposure (Aupinel *et al.*, 2009). This experiment underlined the variability in results and the importance of further investigation of factors such as colony origin of larvae, effect of season and larval heterogeneity at grafting.

In this paper, we give an overview of existing *in vitro* protocols, present the crucial points of rearing honey bee larvae in the laboratory, and discuss where further research or standardization of methods might be useful or necessary. The aim of this paper is not to present a rigorous recipe for rearing larvae *in vitro* (as these are mostly well documented in individual papers), but rather to discuss advantages and disadvantages of different protocols and give recommendations where necessary and meaningful.

2. Larval nourishment

Honey bee larvae do not feed on a readily available vegetal food; rather, they are fed by adult bees. In a colony, worker larvae are fed worker jelly according to their age by their adult sisters. This fact prevented artificial rearing for a long time. A larva is frequently inspected and progressively fed 135 - 143 times with worker jelly during its larval development (Lindauer, 1952; Brouwers *et al.,* 1987). According to a rough estimation, one worker larva is fed a total of 59.4 mg of carbohydrates and 25-37.5 mg of protein (Hrassnigg and Crailsheim, 2005).

The composition of jelly fed to workers has been analysed and compared to royal jelly (food fed to immature queens, see Section 5.10. Royal jelly). Findings suggest that royal jelly and the jelly of worker larvae up to the age of three days are similar, at least regarding protein, sugar, and lipid content (Rhein, 1933; Brouwers *et al.*, 1987). Pantothenic acid, for example, was found to be five times higher in royal jelly than in worker jelly; no other differences in vitamin content were reported (Rembold and Dietz, 1965).

Worker jelly is produced by the hypopharyngeal and mandibular glands of nurse bees (Jung-Hoffmann, 1966; Moritz and Crailsheim, 1987). Biochemical analyses show a broad variation among season, age of fed larvae, investigation methods and colony supply (Jung-Hoffmann, 1966; Brouwers, 1984). According to Kunert and Crailsheim (1987), worker jelly is composed of 39.2-53.3% protein dry weight for larvae younger than four days. After that age, the protein content decreases to 15.7-26.1% dry weight. Correspondingly, the sugar content increases from 7.8-19.6% to 32.2-64.6% dry weight after day four (Fig. 1). These findings were confirmed by Brouwers *et al.* (1987), who moreover described the decrease of lipids in the food of worker larvae with increasing age. Asencot and Lensky (1988) measured the fructose / glucose ratio of worker jelly and also confirmed the increase of sugar content with age.

Four- and five-day-old worker larvae are additionally fed an increasing, although still small, amount of unprocessed pollen (e.g. Rhein, 1933; Jung-Hoffmann, 1966). After day six, larvae cease feeding and larval cells are sealed by adult workers. During pupation, larvae make use of the material gained during larval nourishment for anabolism and catabolism. The metabolism of glycogen and lipids in particular plays an important role during metamorphosis (Hrassnigg and Crailsheim, 2005).

3. History of rearing honey bee larvae in the laboratory

Isolating worker larvae from adult bees in a hive and rearing them to the adult stage has challenged many early researchers who were mainly interested in honey bee physiology and caste determination.

Fig. 1. Comparison of: **a.** protein and; **b.** sugar content of brood food of young (1-3 d) and old (4-6 d) worker larvae (Data summarized from Kunert and Crailsheim, 1987) and laboratory diet, both percentages of dry weight. Protein decrease and sugar increase are due to the increase in sugar added to diets (Aupinel *et al.*, 2005). Range of content is determined by variation in royal jelly protein (Sabatini *et al.,* 2009) and sugar (Brouwers, 1984) content. Protein and sugar in yeast were excluded from calculations.

Bertholf (1927) kept three-day-old larvae in the laboratory alive for more than two days by feeding them different concentrations of sucrose solution, and Velich (1930) reared them to adults. The first important report of hand-feeding honey bee larvae in the laboratory was published by Rhein (1933). He found differences in the jelly fed to workers and queens (royal jelly) and successfully used royal jelly to rear two- to three-day-old worker larvae to adults. He also recognized the problem of worker-queen intermediates. Due to the focus of research, the consistent production of females belonging to the worker caste was one of the main challenges during these early days of *in vitro* rearing of larvae. Presently, the *in vitro* rearing of larvae is being used as a routine method in pesticide testing and for many applications in honey bee physiology and pathology (Table 1). Hence, the reduction of individual feedings and, thus the work load for the experimenter, is appreciated and presents a challenge to those further developing these methods. In contrast to the numerous progressive feedings of larvae in a colony, larvae in the laboratory are only fed daily or even less frequently, preferably without influencing survival rate (Aupinel *et al.*, 2005; Kaftanoglu *et al.,* 2010).

After initial attempts, many researchers developed methods to rear honey bee larvae in the laboratory by modifying or optimizing the basic diet, which consisted of royal jelly diluted with an aqueous solution of glucose and fructose. Fraenkel and Blewett (1943) discovered the importance of yeast in artificial insect diets for *Drosophila* larvae, but this was later replaced with B vitamins and polyunsaturated fatty acids (Vanderzant, 1974). The use of yeast was also applied to honey bee larval diets, and yeast extract is still in use (Michael and Abramovitz, 1955; Peng *et al.*, 1992; Aupinel *et al.*, 2005) but has been omitted by others without a reduction in individual survival rates (Genersch *et al.,* 2005; Genersch *et al.*, 2006). Yeast has been demonstrated not to be a differentiating agent for female castes, but it may be beneficial because of phagostimulatory or nourishing effects (Rembold and Lackner, 1981; Vandenberg and Shimanuki, 1987). Since *in vitro* rearing can also be completed successfully without the addition of yeast extract, the role of yeast needs to be questioned critically. In addition to using yeast, other attempts have been made to enrich the larval diet, for example with a readily available vitamin formulation (Multibionta; Merck; Herrmann *et al.,* 2008) which did not affect larval weight or mortality significantly.

Michael and Abramovitz (1955), amongst others, developed self-feeding dishes and fed an aqueous honey solution containing 10% dehydrated yeast extract. They were the first to inoculate larvae in the laboratory with European foulbrood. Rearing larvae on diluted royal jelly is "an art", as stated by Weaver (1974), and yielded only small numbers of individuals and also queen-worker intermediates. More information on early *in vitro* rearing of honey bee larvae and the success of different researchers can be found in Jay (1964). These early experiments formed the basis for later protocols, produced valuable scientific information and permitted biological testing of different qualities of royal jelly (Weaver, 1955; Smith, 1959; Mitsui *et al.*, 1964; Asencot and Lensky, 1984). The use of worker jelly (which is also disproportionately labour intensive to harvest compared to royal jelly) was doomed, because survival is low and larvae do not pupate

Table 1. Research topics using laboratory-reared honey bee larvae and selected references.

Research topic	Authors
Caste differentiation	Rhein, 1933; Weaver, 1955; Smith, 1959; Shuel and Dixon, 1960, 1986; Mitsui *et al.,* 1964; Rembold *et al.*, 1974; Weaver, 1974; Asencot and Lensky, 1976, 1984, 1988; Shuel *et al.*, 1978; Rembold and Lackner, 1981; Vandenberg and Shimanuki, 1987; Wittmann and Engels, 1987; Patel *et al.*, 2007; Kucharski *et al.*, 2008; Kamakura, 2011; Shi *et al.*, 2011
Diploid drones	Woyke, 1963; Herrmann *et al.*, 2005
Larval pathogens	Michael and Abramovitz, 1955; Peng *et al.*, 1992, 1996; Brødsgaard *et al.*, 1998, 2000; Genersch *et al.*, 2005, 2006; Behrens *et al.*, 2007, 2010; Jensen *et al.*, 2009; Forsgren *et al.*, 2010; Vojvodic *et al.*, 2011a, 2011b, 2012; Vasquez *et al.*, 2012; Foley *et al.*, 2012
Toxicity	Wittmann and Engels, 1981; Davis *et al.*, 1988; Czoppelt and Remboldt, 1988; Aupinel *et al.*, 2005, 2007a, 2007b, 2009; Medrzycki *et al.*, 2010; Da Silva Cruz *et al.*, 2010; Gregorc and Ellis, 2011; Hendriksma *et al.*, 2011a; Gregorc *et al.*, 2012
Transgenic plants	Malone *et al.,* 2002; Brødsgaard *et al.,* 2003; Hendriksma *et al.*, 2011b, 2012

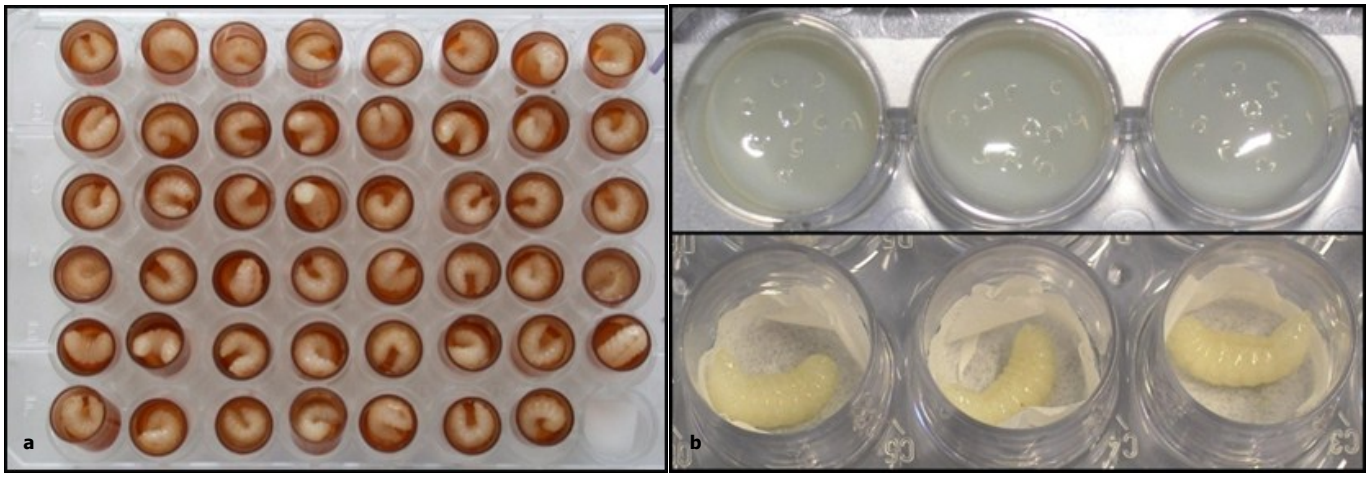

Fig. 2. a. 48-well microtiter plate containing 47 plastic queen cups on dental rolls. Larvae are about 6-7 days old and reared in the same cup from first instar on (Photo: Pierrick Aupinel). ***b.*** 3 groups of 10 each first instars (upper panel) which will be progressively isolated in new wells lined with filter paper, in the end resembling the 3 engorged larvae in one cup each (lower panel, Photo: Elke Genersch).

(Rhein, 1933; Herrmann *et al.*, 2008). Survival on worker jelly can be increased when sugars are added, and Asencot and Lensky (1976; 1984; 1988) believed that sugars together with juvenile hormone could be the queen determinant. More recent results regarding the importance of sugars for pupation are available in Kaftanoglu *et al.* (2011). In turn, to find the caste-determining factor, Rembold *et al.* (1974) reared young worker larvae in thimbles fed a basic food derived from royal jelly via a very extensive alcohol extraction. This diet produced more adult workers, but also more queens and intermediate forms, than did diluted royal jelly. Shuel and Dixon (1986) prepared a complex diet composed of soluble and insoluble protein extracts from royal jelly enriched with solutions containing vitamins, minerals and other compounds.

Wittmann and Engels (1981) suggested investigating the effects of plant protection products on *in vitro*-reared honey bee larvae, and Davis *et al.* (1988) were among the first to administer carbofuran and dimethoate dissolved in royal jelly to honey bee larvae. At the same time, Czoppelt and Rembold (1988) assessed the toxicity of parathion to larvae. Aupinel *et al.* (2005) imitated the age-dependent increase of sugars and dry matter in the larval food in the colony (Brouwers *et al.*, 1987) by gradually increasing glucose, fructose and yeast extract in the diet of larvae (Fig. 1b). They also replaced beeswax cups or thimbles with readily available plastic cups (cupula - used mainly by beekeepers for queen rearing purposes) placed in 48-well microtiter plates (Fig. 2a). This method allows for adoption in testing insecticides in different laboratories (Aupinel *et al.*, 2009).

Two principal *in vitro* rearing methods are employed which we will discuss in this paper. In the first method, one larva is reared per cup and the exact amount of diet a larva consumes daily is administered, following the protocol of Rembold and Lackner (1981) with modifications by Vandenberg and Shimanuki (1987). This amount of diet is estimated to be 160µl in total during larval development (Aupinel *et al.*, 2005) or 164µl for Africanized honey bees (Silva *et al.*, 2009). The second approach administers excess diet, and consequently the larvae have to be transferred to new dishes regularly. At the start of this process several worker (Peng *et al.*, 1992; Genersch *et al.*, 2005; 2006) or drone (Behrens *et al.*, 2007, 2010) larvae are reared in one culture plate well (Fig. 2b). With increasing age, the larvae are either pro gressively isolated by daily grafting on fresh food (Genersch *et al.*, 2005, 2006) or still grafted in groups to new petri dishes (Kaftanoglu *et al.*, 2010). During *ad libitum* feeding, the individual uptake of diet and compounds / pathogens is not restricted to a certain volume (and hence, dosage) as in single-cup protocols. Therefore, it is only possible to determine the LC_{50} (median lethal concentration) for a given substance or pathogen; however, the LD_{50} (median lethal dose) can be roughly estimated (when necessary) by assuming the average consumption to be the abovementioned 160µl per larvae. In many cases it will be sufficient to determine the lethal concentration of a substance or pathogen, because under natural conditions bees and larvae will be exposed to matrices containing a certain concentration of a substance or pathogen. Administering a certain dosage, as is done in medical treatments of humans and other vertebrates, will not be the issue with honey bees, because drugs are administered to bees not in a given dosage per bee but rather dissolved in sugar solution fed to the bee colony. However, it has to be taken into account that all calculated dosage estimates are derived from the assumed consumption, and larvae will consume more if more food is provided. Therefore only individual larval rearing with limited feedings and control of complete consumption allows accurate dosage calculations (Aupinel *et al.,* 2005).

Many of the methods previously published resulted in high mortalities and were labour intensive. However, progress has been made and some of the protocols are sufficient to consistently rear worker honey bee larvae with little or no mortality in the laboratory and can therefore be used for mass rearing and the application of routine testing of compounds (Aupinel *et al.*, 2005; Hendriksma *et al.*, 2011a). This might suggest that no more research efforts to develop

new diets would be necessary. However, researchers need to adapt existing diets adequately for their studies or develop new diets to understand the nutritional requirements of honey bee larvae in detail. There is still no chemically defined diet available, as there are for other insects, and it is unlikely that such a diet will be produced for honey bee larvae (Vanderzant, 1974; Shuel and Dixon, 1986). So far, all published diets have been composed of crude materials (royal jelly), imitating the natural food to a great degree and thus are categorized as a third type of artificial diets as described by Vanderzant (1974).

4. Crucial points for rearing honey bee larvae in the laboratory

4.1. Study design

When testing the effect of plant protection products or insect growth regulators on immature honey bees, the design of the study (i.e. the number of replicates and larvae) is crucial. First, clarity regarding the minimum number of replicates is needed. A replicate is a repetition within a treatment group, commonly made up of a series of microtiter plates or petri dishes, where each larva can be regarded as a single statistical event. We recommend a minimum of three replicates with a minimum of 30 larvae per replicate. In toxicity testing, a replicate typically consists of a control plate without solvent, one control with solvent (if necessary) and several plates with the doses or concentrations of pesticides to be tested. As a reference, one treatment with a substance of known toxicity (e.g. dimethoate, Aupinel *et al.*, 2007a, 2007b) must be conducted (see the *BEEBOOK* paper on toxicology methods (Medrzycki *et al.*, 2013)).

Decisions about study design should carefully consider the aim of the investigation. Depending on the study design, it is possible to study the influence of the genetic background of the bees (Fig. 3a) or of the season (Fig. 3b) on the susceptibility of larvae to a certain substance or pathogen. Mixing larvae from different colonies (Fig. 3c) and testing them even over the entire season (Fig. 3d) will reveal the effect of a substance / pathogen that is not influenced by the genetic background of the bees or by the season. To fully understand the effect of a substance / pathogen, ideally all four approaches should be carried out. If the variance due to an effect of the genetic background needs to be reduced or controlled for, the usage of haploid drone larvae instead of diploid worker offspring of different patrilines may be desirable. This approach however requires that the phenotypes of the drones resemble those of the workers or that differences have been investigated. If usage of diploid workers is inevitable the genetic variance of the study population can be reduced by taking the worker offspring of a single drone inseminated queen. In any case, the minimum required sample size must be calculated and for this we recommend consulting a statistician.

4.2. Caging of queen

- Cage queen on an empty brood comb
- Confirm oviposition by visual inspection after few hours
- 72 hours after confirmation of eggs - Section 5.5. Grafting of larvae

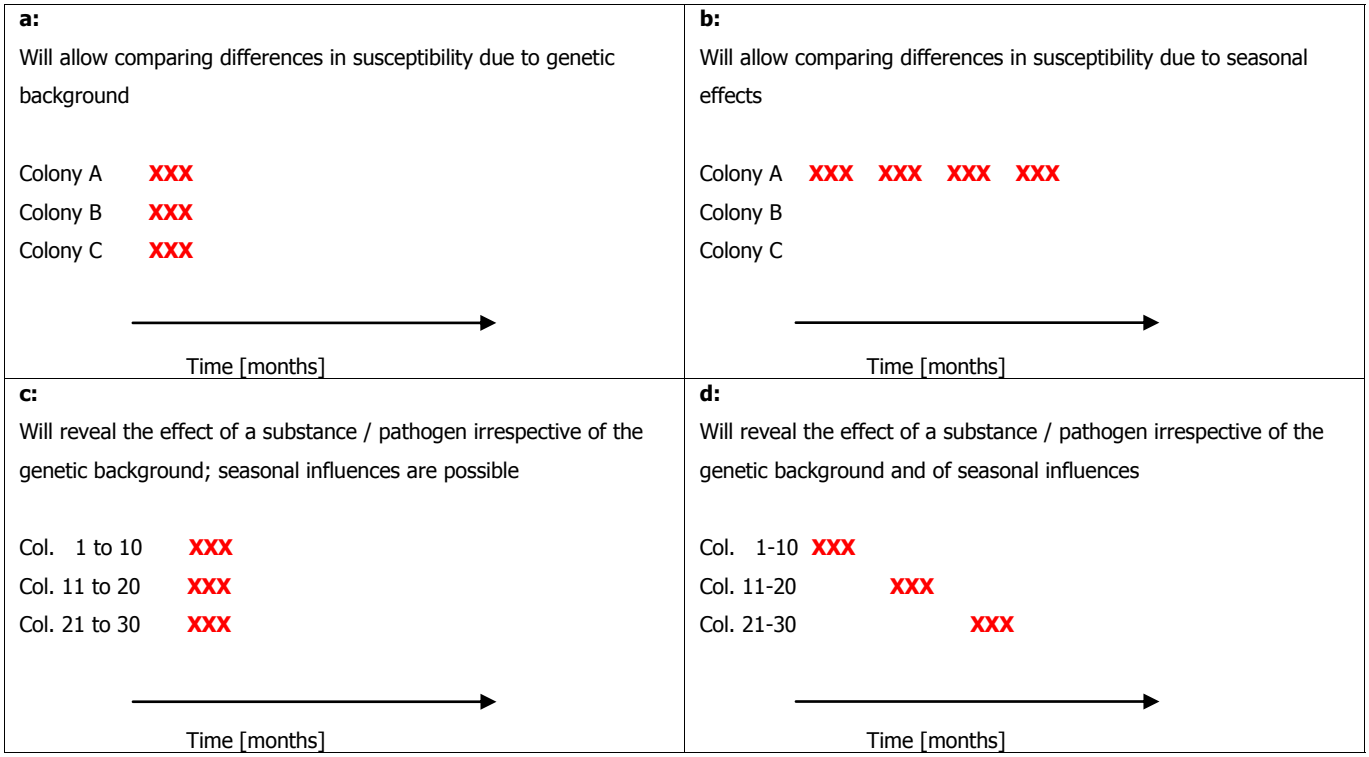

Fig. 3a-d. Examples of four different study designs to conduct three replicates of an experiment with different levels of genetic and seasonal variability. **X** indicates date of grafting and origin of larvae used for experiments.

The developmental stages of honey bee larvae exhibit different susceptibilities towards pesticides and pathogens, and larvae should be exposed to pathogens or toxins in experiments at biologically relevant stages (Davis *et al.*, 1988). If honey bees are to be reared from the very early larval stages, first instar larvae are needed. Two definitions of age are known: the biological age (larval stage) and the chronological age. To differentiate these two morphological parameters need to be investigated. During their development, honey bee larvae undergo five instars, and each instar lasts a different length of time (Bertholf, 1925). Head diameter (Rembold *et al.*, 1980) and the developmental stages of the mouthpart, wing buds, leg buds and the gonapophyses (see drawings in Myser, 1954 and the *BEEBOOK* paper on miscellaneous research methods (Human *et al.*, 2013)) are proper parameters to characterize each of the five instars. As the first instar stage lasts 14-20 hours, one should be aware that a larva that has reached the age of one day (24 hours post hatching from the egg) already has reached the second instar (Bertholf, 1925; Rembold *et al.*, 1980). Larval age can also be determined by weighing (Rembold and Lackner, 1981; Vandenberg and Shimanuki, 1987; Davis *et al.*, 1988). Weight of larvae can be confirmed using data of Wang (1965) and the *BEEBOOK* paper on miscellaneous research methods (Human *et al.,* 2013): 12 hour old larvae have on average a weight of 0.36 ± 0.024 mg but this may depend on honey bee race. However, weighing will delay grafting and increase the risk of contamination and mortality.

Age of first instar larvae for grafting (for the grafting method, see the *BEEBOOK* paper on queen rearing (Büchler *et al.*, 2013) is usually determined chronologically, and larvae younger than 12 hours after hatching are safely first instars. Chronological age can be controlled by caging the queen on a brood comb with a queen excluder cage (for information on obtaining brood and adult bees of known ages, see the *BEEBOOK* paper on miscellaneous research methods (Human *et al.*, 2013)). Queens do not start oviposition immediately after caging, so onset of egg-laying has to be confirmed. It has to be taken into account that larvae hatch from eggs 66 to 93 hours after oviposition (Collins, 2004), hence general time schedules based on 72 hours need to be confirmed for every trial. We recommend that the queen lays eggs in large areas of the comb; larvae of the same age are usually found in ring-like areas on the comb. Releasing the queen from the excluder cage afterwards is important only for colony development and a fixed time cannot be given, as this strongly depends on the queen.

Instead of grafting, larvae can also be obtained using plastic queen cups mounted on commercially available artificial combs (Cupularve Nicotplast, France). On day four after caging the queen and depending on larval hatching, plastic cups containing first instar larvae can quickly be collected (Hendriksma *et al.*, 2011a).

4.3. Sterile environment

- Clean incubator and desiccators
- Sterilize equipment and water for diet preparation
- Sterilize rearing equipment (cups)
- Put plates with cups under UV

When rearing honey bee larvae in the laboratory, strict sanitation is required. A sterile environment with sterile materials and chemicals is crucial; otherwise infections from bacteria or fungi will impede experiments. We recommend laboratories to strive to achieve optimum levels of sterility using their preferred method. All equipment, such as glassware, tools, consumables and water should be autoclaved. Work areas and material for control and exposure / infection treatments should be separated. Autoclaving sugar solution should be avoided because of heat-derived formation of hydroxymethylfurfural (HMF). Two possibilities for the treatment of sugar-yeast-water solutions are recommended: first, to filter with 0.22µm pore membrane filters, and second, to use autoclaved water for the preparation.

Mask and hand disinfection are important to reduce infections. It is crucial to use incubators only for larval rearing, as any other usage needlessly increases the risk of contamination. Disinfect incubators between experiments using ethanol (> 70%) or heat (e.g. more than one hour at more than 100°C) but check operators manual of equipment. As a regular test of sterility, or for troubleshooting, we recommend incubating open agar petri dishes and analysing them to detect any possible sources of contamination. Tools such as paintbrushes (for grafting) must be washed in ethanol and rinsed in autoclaved water before and regularly throughout usage. Dental rolls can be inserted in wells of rearing plates and 500µl of 15.5% glycerol solution added (see 5.8. Incubation conditions). Wet dental rolls and plastic cups can be put under UV-light for sterilization for at least one hour. Methyl benzethonium chloride (MBC) was proposed for the disinfection of plastic cups and also on wetted filter paper or dental roll to prevent microbial growth during incubation (Vandenberg and Shimanuki, 1987; Aupinel *et al.*, 2005). Due to its high price, MBC is no longer in use. In early protocols, methyl-4-hydroxybenzoate was used as a fungicide in the diet (Rembold *et al.*, 1974; Rembold and Lackner, 1981) or Nyastin was added to royal jelly by Herrmann *et al.* (2008). Plastic queen cups can be disinfected in a chlorine solution such as Milton® Sterilising Tablets (Brodschneider *et al.*, 2009). To reduce bacterial infections, Penicillin G (e.g. 375 ppm) can be added to the diet on day one, most of which will degrade within a few days (Riessberger-Gallé *et al.*, 2011).

4.4. Preparation and storage of diets

- Estimate the needed amount of diet
- Prepare or thaw sugar solutions (w/v)
- Mix sugar solutions with royal jelly 1:1 (w/w)
- Warm diet to 34.5°C in the incubator - Section 5.9. Feeding of diets
- Dump remaining diet or store diet maximum 2 days at 4°C

Most artificial diets for honey bee larvae contain royal jelly plus 3-9% (w/v) each of glucose and fructose and 0-2% (w/v) yeast extract (Rembold and Lackner, 1981; Vandenberg and Shimanuki, 1987; Peng *et al.*, 1992; Aupinel *et al.*, 2005; Genersch *et al.*, 2005).

Table 2. Daily amounts and composition for limited feeding according to the age of worker larvae in percentage of weight (Aupinel *et al.*, 2005). For details on preparation of diets (w/v and w/w in particular) see section 5.4.

Day of feeding	Amount [µl]	% D glucose	% D fructose	% yeast extract	% royal jelly
1	10	6	6	1	50
2	10	6	6	1	50
3	20	7.5	7.5	1.5	50
4	30	9	9	2	50
5	40	9	9	2	50
6	50	9	9	2	50

As an example, we present a detailed preparation description for 10 g diet with 6% (w/v) glucose, 6% (w/v) fructose and 1% (w/v) yeast extract (diet A which is fed to larvae on day one according to Aupinel *et al.,* 2005): 1.2 g of glucose, 1.2 g of fructose and 0.2 g of yeast extract are weighed (w) and solved in about 5 ml autoclaved water. This clear solution is filled up to exactly 10 ml (v). For preparing the final diet the aqueous solution must be mixed with fresh royal jelly 1:1 (w/w), for the given example 5 g of each are needed. If frozen royal jelly is used it can be recommended to freeze it in aliquots (e.g. amounts used to feed one plate), ready to be filled up with the same weight (w) of aqueous solution. To optimize worker rearing and avoid intercastes composition of diets and amounts of food must be changed in later larval stages (Table 2).

In another recipe (Genersch *et al.*, 2005; 2006), the larval diet consists of 3% (w/v) glucose, 3% (w/v) fructose, and 66.6% (v/v) royal jelly in sterile, double-distilled water. It is recommended to have a glucose/fructose stock solution containing 9% (w/v) glucose and 9% (w/v) fructose solved in sterile double-distilled water and stored at 4°C until use. Aliquots of royal jelly are stored frozen at -20°C. Every day fresh larval diet is prepared by thawing royal jelly shortly before use and mixing two volume parts of royal jelly with one volume part of glucose/fructose stock solution to obtain the above given concentrations. If an aqueous solution of yeast extract is also added, the concentration of the glucose/fructose stock solution needs to be adjusted to ensure that the larval diet does not contain less than 66.6% (v/v) royal jelly and not more than 33.3% (v/v) aqueous solutions. Otherwise the larval diet will contain too much water and young larvae will tend to drown in the diet or will have problems with digestion.

If investigations start in early spring, no fresh royal jelly will be available; therefore frozen royal jelly has to be used. Also, freeze-dried royal jelly stored at -70°C can be used (Peng *et al.,* 1992). In general, we suggest preparing new diet every day or using prepared and refrigerated diets within three days. Re-freezing should be avoided.

4.5. Grafting of larvae

- Prepare plates
- Apply diets into cups
- Pre-incubate plates and thermoblocks for temperature regulation during grafting
- Take combs to the lab and start grafting
- Section 5.8. Incubation conditions

According to the chosen method, rearing plates or petri dishes have to be prepared in advance, because larvae are already grafted into the first day's diet. Grafting is the collection of larvae from a comb in the same way as beekeepers graft larvae for queen breeding (see the *BEEBOOK* paper on queen rearing for a detailed explanation of grafting (Büchler *et al.,* 2013). A grafting tool or a paintbrush and a binocular loupe are recommended. The first priority in grafting is not to injure larvae. If the first grafting try fails (i.e. one suspects that the larva is injured), another larva should be taken. Larvae lie on their side in the bottom of the comb cell. Consequently, they breathe from the spiracles located on the upper side of their body. Upside-down grafting (i.e. larvae flipped over during the grafting process) should be avoided, because larvae should remain in the same position in the queen cup as originally on the comb due to their use of the open spiracles. Grafting should not take longer than 20 minutes per 48 larvae and a warm (> 20°C, place pre-incubated thermoblocks below rearing plates, see 5.9. Feeding of diets) and humid environment should be maintained throughout. While grafting from one comb, additional brood combs should be stored in an incubator at 34.5°C and more than 60% RH.

4.6. Exclusion of grafting effect

A commonly used option, depending on and if justified by the research aim, may be the exclusion of grafting effects by excluding dead larvae from the experiment 24 hours after grafting. However, this is impossible if susceptibility to a certain substance or pathogen decreases with increasing age, as is the case with *Paenibacillus larvae*, the etiological agent of American Foulbrood. If experiments can be performed equally well with second or older instar larvae, then it might be more appropriate to graft the more robust second or older instar larvae right away instead of replacing larvae in the course of the experiment. In acute toxicity testing, sometimes a surplus of larvae is used at day four to replace larvae which died prior to treatment administration. Of course, this procedure increases the quality of the resulting honey bees and thus biases results. Whether or not to replace larvae during the experiment must be considered carefully prior to use, explicitly documented, and critically discussed when evaluating the results.

4.7. Randomization

Random application of treatments to test larvae may be used to improve dose-effect curves in toxicity studies (Pierrick Aupinel, unpublished data). We recommend randomizing larvae within a study in order to avoid an eventual bias due to the minor differences of age and then a difference of susceptibility to the test pathogen or compound. Randomization can be conducted at different moments according to

the applied protocol. Randomization is easily accomplished when determining the acute toxicity of a test compound. For example, one can randomly exchange queen cups on microtiter plates on day four, just before one ordinarily provides larvae with contaminated diet in an acute toxicity test. If testing in chronic conditions, cup randomization must be done at the grafting stage because larval exposure to test compounds begins at this moment. Larvae can be randomly dispatched directly from the comb to the plastic cups.

4.8. Incubation conditions

- Clean environment is needed (Section 5.3. Sterile environment)
- Prepare saturated solutions of K_2SO_4 and NaCl, place in open dishes in desiccator
- Incubate larvae at 34.5°C and 95% RH for the first 6 days
- On day 7 change humidity to 80% RH

Honey bee larvae may be kept in hermetic plexiglass desiccators or other air-tight plastic containers (to facilitate humidity control, e.g. Tupperware) placed inside incubators. Incubators alone are also adequate when the necessary humidity can be maintained in the chamber. It is important to maintain the desired rearing temperature of 34.5°C with maximum precision (± 0.5°C), as suboptimal larval temperature affects adult bee longevity and adult bee resistance to dimethoate and induces malformed wings (Medrzycki *et al.,* 2010). Furthermore, pathogens may increase or lower in pathogenicity according to temperature (Vojvodic *et al*., 2011a). The deviation of incubation temperature must be kept as low as possible and temperature should be verified with data loggers to help explain any possible problems with mortality. Beside temperature, honey bee larvae also require constant and high humidity (Human *et al.,* 2006). Most researchers propose a humidity of 95-96% RH during the first 6 days followed by a reduction to 70-80% RH, which proved to be appropriate (Rembold and Lackner, 1981; Vandenberg and Shimanuki, 1987; Peng *et al.,* 1992; Aupinel *et al.,* 2005). The humidity adjustment is accomplished by first placing a dish with a saturated solution of K_2SO_4 (to achieve 95% RH) and later a saturated solution of NaCl (to achieve 80% RH) on the bottom of the desiccator. More on preparation of these solutions can be found in section 6.3 "Relative Humidity" of the *BEEBOOK* paper on maintaining adult bees in the laboratory (Williams *et al*., 2013). Humidity should also be measured with data loggers regularly to verify accuracy. If glycerol soaked dental rolls are used (see 3. Sterile environment) they have to be removed at day 7 (Aupinel *et al.,* 2005). Alternatively, good humidity results have been obtained with a much easier approach by just filling up to one fourth of the wells of the plate with water, closing the plate with the accompanying lid, and placing the plates in bacterial incubators, i.e. incubators without CO_2 and humidity adjustment (Genersch *et al*., 2005, 2006).

We recommend protecting larvae and reducing the handling of their rearing dishes to reduce mortality. The exposure of larvae to temperature and humidity conditions other than those in the incubator increase bacterial or fungal infections and mortality. Larvae should be weighed only if necessary for experiments. Transferring to new dishes or carefully cleaning larvae at the beginning of or after defecation (Smith, 1959; Shuel and Dixon, 1986, Genersch *et al.,* 2005, 2006) is not performed in many methods, although it is reported to increase successful pupation and reduce pupal mortality (Vandenberg and Shimanuki, 1987).

4.9. Feeding of diets

- Warm diet to 34.5°C in the incubator
- Place rearing plates on pre-incubated thermoblocks
- Check mortality - Section 5.12. Assessing survival
- Remove dead larvae
- Place food by using a pipette next to the head of each larvae

Feedings can either be administered every day (for 6 days) in different amounts or, if allowed by the study design, the first two portions (10 + 10µl) can be administered the first day. This option, recommended by Aupinel *et al.* (2005), gives one day off from the lab and it is assumed that this does not affect mortality or the quality of the test. The total amount of diet given to each larva is 160µl and should be reported if altered. When feeding 160µl of diets in total, honey bee larvae will consume all the food administered: therefore the cleaning of plastic cups or transfer to new cups is not necessary, as each manipulation increases mortality. Prior to feeding, the diet is carefully heated on a magnetic stirrer or in incubators to 34.5°C. During the feeding procedure, the pipette should be placed on the inner side of the plastic cup and the food drop should be placed next to the mouth of the larvae. Drowning of the larvae should be avoided. If drowning occurs occasionally, lift the larvae with a sterile paintbrush; if it occurs systematically, check method (e.g. humidity inside the desiccator or water content of diet).

When larvae are reared following a protocol implying feeding *ad libitum*, each well is filled with an appropriate volume of diet depending on the volume of the well (e.g. 500µl per well of a 24-well plate), and larvae are then added to the well by placing them carefully on top of the larval diet. Larvae are transferred to newly filled wells every day and the number of larvae per well is adjusted to accommodate the size of the growing larvae. While ten first instar larvae can easily be placed into one well of a 24-well plate, each engorged larva needs a separate well (Fig. 2b). When using this rearing protocol, it is essential to remove any dead larva from the wells and to not transfer dead larvae into newly filled wells. Shortly before pupation, i.e. shortly before or after defecation, larvae are gently cleaned from adhering food and faeces by carefully rolling them over tissue paper (Kimwipes) and then transferred into pupation wells lined with filter paper (Peng, 1992; Genersch *et al.,* 2006). If defecation continues in the pupation-well, developing larvae are placed in new wells lined with clean filter paper.

It is important to pay attention to temperature during feeding. Thermoblocks (pre-warmed in incubators at 34.5°C) underneath the rearing plates can be used to stabilize the temperature of the larvae.

4.10. Royal jelly

- Check quality of royal jelly (preliminary experiments)
- Store royal jelly in aliquots at -20°C until usage
- Thaw royal jelly briefly before (Section 5.4. Preparation and storage of diets)

Royal jelly is the brood food produced by nurse bees to specifically feed queen larvae. The immature queen is superabundantly fed, and older queen larvae are fed a different formation than worker larvae (Brouwers *et al.*, 1987). This jelly is harvested by beekeeping operations and commercially available. Due to its manifold use, including for humans, much research on quality standards of this undefined hive product has been conducted. Royal jelly makes up a great part of the larvae's laboratory diet; thus its quality strongly influences *in vitro* rearing success. The exact composition of royal jelly and the importance of several of its components for larval development are not clear. Fresh royal jelly contains roughly 9-18% protein, 7-18% sugars and 3-8% lipids (Sabatini *et al.*, 2009). The water content varies between 50 and 70% (Rembold and Dietz, 1965; Sabatini *et al.*, 2009; Zheng *et al.*, 2011). Royal jelly contains 10-hydroxy-2-decenoic acid (10-HDA), an antibacterial substance that is analysed as a freshness parameter in routine testing.

The biochemical composition of royal jelly depends on harvesting season and regional origin (Sabatini *et al.*, 2009). Moisture and protein content also depend on the harvesting day (i.e. first, second, third) after grafting of larvae (Zheng *et al.*, 2011). The latter fact has long been demonstrated to influence the development of ovaries of *in vitro* -reared larvae (Mitsui *et al.*, 1964). Accordingly, differences even from several batches of royal jelly may significantly alter the quality (e.g. protein content, see Fig. 1) of *in vitro* diets and influence rearing success or susceptibility against pathogens. The quality of royal jelly is presumably also altered by shipping and storage condition and duration. Differences in freeze-dried, frozen or fresh royal jelly (harvested during investigation year) can be expected. For experiments involving the testing of pathogens, the presence of unwanted antibiotics in royal jelly must be excluded, hence we recommend the use of organic royal jelly or own production. Royal jelly may also be irradiated at 20 kGy for purposes of sterilization. Though it is not known if this will impact its structural integrity or affect its developmental impact on larvae, Gregorc and Ellis (2011) and Gregorc *et al.* (2012) reared larvae successfully on irradiated royal jelly. It is advisable to test every new batch of royal jelly to make sure that the results obtained with the 'old' batch can be reproduced with the 'new' batch. Sometimes it might be necessary to test several batches before a batch suitable to replace the consumed charge can be identified.

4.11. Application of test substances

Substances (toxins or pathogens) can be tested in chronic or acute conditions. In chronic testing, a substance is mixed into the diets at a constant concentration and provided to the larvae at each feeding day. Acute exposure can occur at every feeding day by mixing the tested substance into the diet once. The testing compound is dissolved in water. If it is not soluble in water at the experimental concentrations, other solvents such as acetone can be used. In that case, it is required to have a second control group fed with diet containing the solvent at the same concentration as the treated samples. The proportion of the solvent must not exceed 10% of the final volume. A different rate has to be justified. In all cases, a constant volume for the different dilutions must be used in order to maintain a constant concentration between the control diet and the test substance diet. In order to assess the adequate LD_{50} or LC_{50} range, it is recommended to run a preliminary experiment where doses of the test substance may vary according to a geometrical ratio from 5 to 10 (for examples, see the *BEEBOOK* paper on toxicology methods (Medrzycki *et al.,* 2013)).

4.12. Assessing survival

Larvae can be classified as dead when respiration ends, when they lose body elasticity, or when they develop oedema and change colour to greyish or brownish (Genersch *et al.*, 2005, 2006). Dead larvae should be removed from the incubator to prevent decomposition by bacterial or fungal saprophytes and subsequent contamination of other larvae; in pathology studies dead larvae need to be sampled anyway. Non-emerged individuals on day 22 after grafting are counted as dead during pupal stage. Live adults and dead adults that have left their cell and show a regular development are both counted upon emergence or at the latest at day 22.

4.13. Control mortality

If *in vitro* larval rearing methods are supposed to become a commonly accepted method to test plant protection products, the maximum tolerated control mortality must be considered for validation. Techniques have improved over the years, and control mortality has progressively reached low levels. Nonetheless, control mortality has to be reported regularly, to demonstrate successful trials. Fukuda and Sakagami (1968) found in a normal colony setting, that 85% of adult workers emerge from the eggs laid by a queen, with mortality occurring in all premature stages. This data suggest a natural mortality of about 15% during larval and pupal development. Therefore, in control samples of *in vitro*-reared larvae, total mortality until late pupal development (~ day 14 after grafting of first instar larvae) should be lower or equal to 15%. This is especially important for the assessment of the median lethal dose (LD_{50}) or the median lethal concentration (LC_{50}), while 20% control mortality can be accepted for the assessment of a NOAEL (no observable adverse effect level) or a NOAEC (no observable adverse

effect concentration). In case of higher mortality in control samples, the replicate is invalidated or convincing reasons, like adverse weather conditions before grafting of larvae or late season, must be given. However, when *in vitro* rearing of larvae is just one method among others to answer a scientific question, we assume a control mortality of 5% from grafting to day 7 to be very good, and 10% tolerable; from grafting to day 22, 20% is very good, while 25% can be tolerated. Care should be taken in presenting these parameters unambiguously and the way they were calculated. These levels should be achieved without exclusion of the grafting effect (see 5.6. Exclusion of grafting effect). Several research institutes have already accomplished control mortalities below these suggested ones (Aupinel *et al.,* 2005; Aupinel *et al*., 2009; Brodschneider *et al*., 2009; Hendriksma *et al.,* 2011a; Kaftanoglu *et al.,* 2010, 2011).

4.14. Capping

In order to investigate parameters of adult honey bees, capping of the rearing plates with perforated bees wax and turning the plates to a vertical position on day eleven has been proven as a useful add-on (Riessberger-Gallé *et al.,* 2008; Brodschneider *et al.,* 2009). A wax layer big enough to cover one 48 well rearing plate is obtained by squeezing about 20 cm^2 of soft (warm) but not liquid comb foundation between two sheets of paper. The bees are able to bite through the thin, almost transparent, wax layer, which allows for the use of the eclosion rate as an additional parameter of survival. Rotating the rearing plates not only reduces malformations due to the unnatural vertical position of larvae in horizontal rearing plates, but capping may also improve microclimate (humidity) in cells and act as an additional barrier against fungi.

4.15. Evaluation of rearing success

The control mortality for evaluation of rearing success was already discussed (see 5.13. Control mortality). Researchers also measured physiological parameters such as body weight, body size, ovary development and flight performance to evaluate rearing methods. A comparison with sisters naturally reared in a colony provides information about the quality of the artificial diet and rearing condition. This comparison reveals that larvae reared in the laboratory are close to those reared in a colony. They have slightly reduced wing areas and thorax dry weights, but reach the age of foragers when introduced into a colony and engage in long persisting flights similar to those of naturally reared control bees (Brodschneider *et al*., 2009).

Another problem is the predictable production of individuals unambiguously belonging to the worker caste, which might be necessary for certain scientific questions. Early attempts to rear worker larvae in the laboratory frequently resulted in inter-castes (Rhein, 1933; Weaver, 1974; Rembold *et al.,* 1974; Rembold and Lackner, 1981; Shuel and Dixon, 1986). Therefore, and also because larval nutrition affects ovary development (Hoover *et al*., 2006), the developmental stages of ovaries and number of ovarioles of laboratory-reared honey bees have been investigated (Weaver, 1955; Mitsui *et al*., 1964; Kaftanoglu *et al*., 2010). Also other characteristics such as developmental time, tongue length, spermatheca, chaetotaxy of metathoric legs, sting lancet, mandibular notch, weight at adult emergence and juvenile hormone titer are used for differentiation (Weaver, 1974; Asencot and Lensky, 1976, 1984; Shuel and Dixon, 1986; Kucharski *et al.,* 2008; Kamakura, 2011).

4.16. Rearing reproductives in the laboratory

According to investigations on honey bee workers, there are many reports on rearing queen bees in the laboratory (Hanser, 1983; Patel *et al.,* 2007). Most recently, Kamakura (2011) produced queens feeding just one basic diet with 6% glucose, 6% fructose and 1% yeast extract, and no increase of matters in the following diets. Amounts of diets and reason for queen development are not given. The same plastic cups can be used, as they are also applied in queen breeding in the colony.

In vitro rearing of drone larvae is also possible feeding previously established worker diets at least up to the prepupal phase (Herrmann *et al*., 2005; Behrens *et al.,* 2007, 2010) and even up to adult emergence (Woyke, 1963; Takeuchi *et al*., 1972). However, in some cases the control mortality in drones during in vitro rearing was higher than in workers. This could be due the fact that drone larvae might in fact need a slightly different food composition than workers (Hrassnigg and Crailsheim, 2005) which has not been studied in detail yet. But also the longer developmental time (especially during the very critical pupal phase) or lethal factors that become visible in the haploid genome might play a role. Clearly, more work is needed to obtain results in drones that are comparable to those of workers and queens. Especially the timing of changing diets would need to be adapted to the development of drone larvae.

5. Discussion

Rearing honey bee larvae *in vitro* is of great importance for research on pathogens and risk assessment. Recent studies have developed protocols that gained success in producing unambiguous workers with low mortalities and facilitated the use of readily available materials (Vandenberg and Shimanuki, 1987; Aupinel *et al.,* 2005). This enables laboratories to rear high numbers of workers compared to the significant efforts from pioneer studies (Rhein, 1933). However, the quality of food, incubation conditions and occurrence of infections can also result in high mortality, and we therefore present some recommendations to reduce undesirable effects.

The production of honey bee workers in the laboratory is still a challenge, in contrast to queen breeding. Queen breeding (see the *BEEBOOK* paper on rearing queens (Büchler *et al*., 2013) has been

employed by beekeepers for decades. It is a process in which larvae grafted into queen cups are raised to queens in a colony, and has also been employed in the laboratory, where larvae are grafted on royal jelly *in vitro*. In all described methods royal jelly is diluted to reduce its queen-potency and produce workers. Alternatively, the recent findings regarding 'Royalactin' may provide another possibility. According to Kamakura (2011), 'Royalactin', a 57 kDa protein, is the queen-determining factor in royal jelly. He demonstrated that not only worker larvae, but even *Drosophila* larvae fed on the synthesized protein exhibit features normally associated with the honey bee queen: shortened developmental time, enhanced longevity and increased egg production. Even more intriguing is the fact that this protein seems to degrade with storage, and honey bee larvae reared on royal jelly stored at 40°C for 30 days all develop with a full worker morphotype. The potential of these findings to allow mass rearing of workers on degraded royal jelly needs further attention and investigation.

We gave much detailed information on what must or can be taken into account to make *in vitro* rearing of honey bee larvae comparable among different laboratories. Researchers also need the freedom to adopt the given recommendations as needed but in accordance with their research purpose and time management. In the end, researches must carefully consider a method (e.g. larval grafting stage, single or group rearing, *ad libitum* or limited feeding of diets or quantity and quality of manipulations) that is justified by the research topic.

In conclusion, it is absolutely essential for the successful rearing of honey bee larvae to use fresh materials of high quality and to gain experience in the chosen method before applying it to any scientific question or to routine testing of compounds and pathogens. However, even with the best of all methods, *in vitro*-reared larvae will always be *in vitro*-reared larvae and never become indistinguishable from larvae reared in a healthy colony. Therefore, researchers have to remain critical regarding results obtained with *in vitro*-reared larvae. Researchers need to decide how close to 'natural' (meaning reared in a colony, *in vivo*) bees they want to come with their *in vitro* efforts and adjust efforts accordingly. In general, the similarity of bees produced by current protocols to natural bees has been proven. This is a strong argument for the use of these methods in research and as the official testing method for plant protection products. Like with all other *in vitro* methods, these larvae are, although a valuable research tool, just a model for reality rather than reality itself.

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