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REVIEW ARTICLE

Standard methods for maintaining adult *Apis mellifera* in cages under *in vitro* laboratory conditions

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Summary

Adult honey bees are maintained *in vitro* in laboratory cages for a variety of purposes. For example, researchers may wish to perform experiments on honey bees caged individually or in groups to study aspects of parasitology, toxicology, or physiology under highly controlled conditions, or they may cage whole frames to obtain newly emerged workers of known age cohorts. Regardless of purpose, researchers must manage a number of variables, ranging from selection of study subjects (e.g. honey bee subspecies) to experimental environment (e.g. temperature and relative humidity). Although decisions made by researchers may not necessarily jeopardize the scientific rigour of an experiment, they may profoundly affect results, and may make comparisons with similar, but independent, studies difficult. Focusing primarily on workers, we provide recommendations for maintaining adults under *in vitro* laboratory conditions, whilst acknowledging gaps in our understanding that require further attention. We specifically describe how to properly obtain honey bees, and how to choose appropriate cages, incubator conditions, and food to obtain biologically relevant and comparable experimental results. Additionally, we provide broad recommendations for experimental design and statistical analyses of data that arises from experiments using caged honey bees. The ultimate goal of this, and of all COLOSS *BEEBOOK* papers, is not to stifle science with restrictions, but rather to provide researchers with the appropriate tools to generate comparable data that will build upon our current understanding of honey bees.

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Métodos estándar para el mantenimiento de adultos de *Apis mellifera* en cajas bajo condiciones de laboratorio *in vitro*

Resumen

Las abejas adultas se mantienen *in vitro* en cajas de laboratorio para una variedad de propósitos. Por ejemplo, los investigadores pueden realizar experimentos con las abejas de miel enjauladas individualmente o en grupos para estudiar aspectos de la parasitología, toxicología y fisiología en condiciones muy controladas, o pueden meter en las cajas panales completos para obtener obreras recién emergidas de cohortes de edad conocida. Independientemente del propósito, los investigadores deben manejar una serie de variables, que van desde la selección de los sujetos a estudiar (por ejemplo, la subespecies de abeja), al ambiente experimental (por ejemplo, temperatura y humedad relativa). Aunque las decisiones tomadas por los investigadores no tienen por qué poner en peligro el rigor científico de un experimento, si que pueden afectar profundamente a los resultados, y pueden dificultar las comparaciones con estudios similares pero independientes. Centrándonos principalmente en obreras, ofrecemos recomendaciones para mantener adultos en condiciones de laboratorio *in vitro*, si bien reconocemos algunas lagunas en nuestro conocimiento que requieren una mayor atención. En especial, se describe cómo obtener correctamente abejas, y cómo elegir cajas adecuadas, las condiciones de incubación, y los alimentos para obtener resultados experimentales biológicamente relevantes y comparables. Además, ofrecemos recomendaciones generales para el diseño experimental y el análisis estadístico de los datos que surgen de experimentos con abejas enjauladas. El objetivo final de éste, y de todos los artículos de *BEEBOOK* y COLOSS, no es limitar la ciencia con restricciones, sino más bien proporcionar a los investigadores las herramientas necesarias para obtener datos comparables que se basen en el conocimiento actual de las abejas melíferas.

实验室条件下笼中饲养成年西方蜜蜂的标准方法

很多研究都需要在实验室内应用蜂笼饲养成年蜜蜂，比如，研究者可能应用单个蜂笼或多个蜂笼开展严格控制条件下的寄生虫学、毒理学或生理学研究。也可能把整个巢脾关入笼中来得到日龄明确的刚羽化出房的蜜蜂。不管目的如何，研究者必须控制多个变量：从研究对象（不同的蜜蜂亚种）到实验环境（温度和相对湿度等。虽然研究者的选择可能不一定会损害实验的科学性，但可能会显著影响实验结果，使独立实验成为相关实验。围绕饲养工蜂，我们推荐了在实验室条件下饲养成年蜂的方法。特别描述了如何恰当的饲养蜜蜂以及如何选择饲养笼、温箱和饲料以得到具生物学意义并具可比性的实验结果。此外，针对实验设计和数据的统计分析还给出了大量建议。本文以及本书中所有文章所涉及的研究方法，其最终目的是给研究者提供合适的研究工具，得到具有可比性的数据，推进我们对蜜蜂的认识，而不是设立技术障碍，限制科学发展。

Keywords: *Apis mellifera*, honey bee, colony losses, hoarding, cage, *in vitro*, laboratory, COLOSS, *Nosema*, toxicology, *BEEBOOK*

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1. General introduction

Recent dramatic losses of honey bee (*Apis mellifera*) colonies in many regions of the world are primarily attributed to introduced and native parasites and diseases, environmental toxins, genetic constraints, beekeeper management issues, and socio-economic factors, acting singly or in combination (Neumann and Carreck, 2010; vanEngelsdorp and Meixner, 2010; Williams *et al.*, 2010). We can study potential effects of many of these factors at the colony-level under field or semi -field (e.g. in tunnel tents) conditions, or at the individual or small group level in a laboratory under relatively controlled settings using honey bees isolated from the outdoors.

Regardless of purpose, maintaining adult honey bees *in vitro* in the laboratory prior to or during experiments is often required, and in many cases can provide better control of extraneous variables. For example, host-parasite interactions (e.g. Forsgren and Fries, 2010), parasite management products (e.g. Maistrello *et al.*, 2008), toxicology (e.g. Johnson *et al.,* 2009) and physiology (e.g. Alaux *et al.*, 2010) can be studied. Honey bees can also be caged individually for the evaluation of learning and memory using techniques such as the proboscis extension reflex (e.g. Frost *et al.*, 2011, 2012; Giurfa and Sandoz, 2012).

Here we discuss important factors that researchers must consider when maintaining adult worker honey bees under *in vitro* conditions in the laboratory using cages that restrict movement to the surrounding outdoor environment. We also briefly describe the maintenance of queens and drones. Because an individual's condition can have profound effects on experimental results, it is vital that adults be maintained under appropriate, controlled conditions that enhance repeatability of experiments. Ultimately, our discussions and recommendations presented here are aimed at facilitating and standardising general care of workers in the laboratory for use in scientific investigations. Additional and more specific information on laboratory methods and settings best suited for the purpose of one's study can be found in greater detail in other parts of the COLOSS *BEEBOOK* (Williams *et al.* 2012), such as in the nosema (Fries *et al.*, 2013), toxicology (Medrzycki *et al.,* 2013), larval rearing (Crailsheim *et al.,* 2013), and behaviour (Scheiner *et al.*, 2013) papers of the COLOSS *BEEBOOK*.

2. Experimental design

2.1. Important experimental design considerations before caging adult workers in the laboratory

Although this paper discusses maintenance of adult worker honey bees in a laboratory outside of a colony, regardless of study type or purpose, it is important to highlight that careful consideration needs to be given to experimental design and statistical analysis of the ensuing data before any practical work should commence. Importantly, one must determine if sufficient resources are available to perform rigorous research with an appropriate level of reproducibility; if constraints preclude good science, it may not be worth conducting experiments in the first place.

General recommendations for design of experiments and analysis of data can be found in the *BEEBOOK* paper on statistical methods (Pirk *et al.,* 2013).

2.2. Independence of observations for laboratory cage experiments involving adult workers

A fundamental aspect of good experimental design is independence of observations; what happens to one experimental unit should be independent of what happens to other experimental units before results of statistical analyses can be trusted.

Until shown otherwise, workers within the same cage are not independent, so each cage becomes the minimum unit to analyse statistically (i.e. the experimental unit). Caging workers individually is therefore extremely desirable because each honey bee can be considered to be an independent experimental unit. Although a method for maintaining workers individually for one week exists (section 5.2.3), one that enables individual workers to be maintained in isolation in the laboratory for even longer periods would be beneficial for certain experiments (so long as social interaction is not the focus of investigation or necessary to the phenomenon(a) investigated).

Additionally, careful consideration is required when performing experiments on which volatiles emitted by workers can influence measured parameters. This might require using separate incubators.

2.3. Appropriate worker and cage replicates for laboratory experiments involving adult workers

A minimum sample of 30 independent observations per treatment is relatively robust for conventional statistical analyses (e.g. Crawley, 2005); however, financial constraints and large effect sizes (e.g. difference among treatments for the variable (s) of interest; see statistics paper (Pirk *et al.* (2013)) will no doubt lower this limit, especially for experiments using groups of caged workers. Larger sample sizes (i.e. number of cages and workers per cage) reduce the probability of uncontrolled factors producing spurious insignificance or significance, and help to tease apart treatments with low effect size. Repeated sampling of individuals over time to observe development of parasite infection, for example, will also require larger samples.

Furthermore, it is important to consider biological relevance of the numbers of individuals in each cage. Unsurprisingly, isolated workers die much quicker than those maintained in groups, possibly due to timing of food consumption (Sitbon, 1967; Arnold, 1978), so experimenters must be aware of expected duration of survival. Possible individual and social behaviours that are of interest should also be considered (e.g. Beshers *et al.,* 2001). For example, > 75 workers were needed to consistently elicit clustering behaviour (Lecomte, 1950), whereas 50 workers and a queen were needed for the initiation of wax production (Hepburn, 1986).

A Monte Carlo simulation model incorporating average lifespan (and standard deviation) for treatments and controls has been created to determine percentage of cases where a significant difference is obtained between groups. Without preliminary trials to determine the magnitude of an effect elicited by an experimental treatment as well as the variation between cages in that effect, statistical power may be impossible to know in advance. In such cases, it is advisable to maintain as many cages per treatment (≥ 3) and individuals per cage (≥ 30) as possible. Examination of the literature for similar studies may also help choose sample size; however, caution should be exercised due to differences in experimental conditions. Refer to the *BEEBOOK* paper on statistical methods (Pirk *et al.*, 2013) for further details on the Monte Carlo simulation and on selecting appropriate sample sizes.

2.4. Appropriate randomisation of study organisms for laboratory cage experiments involving adult workers

When designing studies it is crucial that experimenters avoid bias when choosing study subjects. Workers, for example, can exhibit significant genetic variation for expression of mechanical, physiological, immunological, and behavioural responses used in disease resistance (Evans and Spivak, 2010). This diversity can occur among workers in the same colony or among honey bees from different geographic regions. Additionally, timing and method of collection, as described in section 4, can also have a significant influence on results. Because of this, careful consideration is needed when choosing colonies from which to collect experimental honey bees. To promote a repeatable investigation that is representative of a honey bee population in question, workers should be collected from as many, and as diverse a set of, colonies as possible. It is generally recommended to randomly mix workers from all source colonies among all cages during a study to minimize potential colony-level effects on experimental results. Refer to the *BEEBOOK* statistics paper by Pirk *et al.* (2013) for details on determining number of colonies from which to source individuals and for how to properly randomize individuals and cages for experiments, as well as section 4 for choosing and obtaining workers for experiments.

3. Statistical analyses

Specific details on statistical analyses of honey bee experimental data can be found in the statistics paper of the *BEEBOOK* (Pirk *et al.,* 2013) or in statistical texts.

3.1. Where the response variable is not mortality during laboratory experiments involving adult workers

If a response variable to be measured (e.g. a phenotype of interest that may change with treatment) is quantitative or qualitative (i.e. diseased versus not diseased), then a generalised linear mixed model (GLMM) can be used to analyse data in which 'cage' is a 'random effect' parameter and treatment is a 'fixed effect' parameter (Crawley, 2005; Bolker *et al.*, 2009). Several fixed and random effect parameters can be analysed in the same statistical model. If individuals in two or more experimental cages used in the same treatment group are drawn from the same colony, then a GLMM with 'source colony' as a random effect parameter should also be used to analyse data. This random effect accounts for the fact that, within the same treatment, variation between two cages of honey bees drawn from the same colony may not be the same as variation between two cages drawn from two separate colonies. This statistical approach accounts for the problem of pseudoreplication in experimental design. If the factor 'cage' and 'source colony' are non-significant, an experimenter may be tempted to treat individual honey bees from the same cage as independent samples (i.e. ignore 'cage'). Logically, however, workers drawn from the same cage are not truly independent samples and therefore it would inflate the degrees of freedom to treat individual workers as individual replicates. This point requires further attention by statisticians. In lieu of an immediate solution to this statistical issue, an experimenter can consider using a nested experimental design in which 'individual honey bee' is nested within 'cage', as presented above.

3.2. Where the response variable is mortality during laboratory experiments involving adult workers

If survival of workers is the response variable of interest, a typical survival analysis can be undertaken, such as the parametric Kaplan-Meier survival analysis for 'censored' data (so-called right-censored data in which bees are sampled from a cage during an experiment) or the non-parametric Cox proportional hazards model (Cox model) for analysing effects of two or more 'covariates', or predictor variables, such as spore intensity of the microsporidian *Nosema ceranae* or black queen cell virus titres (Zuur *et al.*, 2009; Hendriksma *et al.*, 2011).

3.3. Statistical software for laboratory experiments involving adult workers

Numerous statistical computing programmes are available to handle analyses mentioned in sections 3.1 and 3.2, such as the freeware R (R Development Core Team; Vienna, Austria), as well as other packages, including Minitab® (Minitab Inc.; State College, USA), SPSS® (SPSS Inc.; Chicago, USA), and SAS® (SAS Institute Inc.; Cary, USA). See the statistics paper of the *BEEBOOK* (Pirk *et al.,* 2013) for details.

4. Obtaining adult workers for laboratory experiments

4.1. Considerations for choosing and obtaining adult workers for laboratory experiments

Consideration of honey bee material to be used for experiments must be made prior to practical work because environmental and genetic factors can profoundly influence results (e.g. Fluri, 1977; Evans and Spivak, 2010). Here we discuss a number of factors that may influence worker collection for experiments.

4.1.1. Seasonal timing of adult worker collection for laboratory experiments

In temperate climates workers can be classified either as short-lived "summer" or long-lived "winter" individuals. Physiological differences, such as in juvenile hormone and vitellogenin levels (Fluri *et al.*, 1977; Crailsheim, 1990; Seehus *et al.*, 2006; Corona *et al.*, 2007; Strand, 2008), are mainly driven by quantity of protein consumption and level of brood rearing by the colony (Maurizio, 1950; Amdam *et al.,* 2004; 2005b). "Summer" individuals can be collected beginning in late spring, after colonies have replaced old "winter" honey bees, and up until late summer, when colonies start to prepare for winter. For specific experiments in which the susceptibility of winter bees is the object of study, one can cage the queen within the broodnest for greater than 21 days so that the queenright colony contains no brood (Maurizio, 1954; Fluri *et al.,* 1982). This mimics the broodless period experienced by honey bees in temperate climates.

4.1.2. Subspecies of adult workers used for laboratory experiments

Honey bees subspecies can exhibit great morphological, behavioural, physiological, and genetic variation (Ruttner, 1987), with subsequent differences in productive traits and in disease susceptibility (Evans and Spivak, 2010; DeGrandi-Hoffman *et al.*, 2012). The same subspecies of honey bees should be used for an experiment.

If one wants to further limit influence of genetics on experimental results, individuals from a single colony or multiple colonies that are headed by sister queens can be collected. This will, however, limit the ability of experimental findings to be more broadly generalized across the study population compared to studies that obtained experimental individuals from multiple, genetically diverse colonies of the same subspecies. Refer to section 2 on experimental design in this paper, as well as *BEEBOOK* papers by Meixner *et al.* (2013) for characterizing honey bee subspecies and Delaplane *et al.* (2013) for discussions on preparing colonies for experiments.

4.1.3. Age of adult workers used for laboratory experiments

Adult workers differ greatly in their physiology depending on their age. For example, changes in host immune response (Amdam *et al.*, 2005a) and morphology (Rutrecht *et al.*, 2007) over time can result in differences in disease resistance and susceptibility to parasites (Villa, 2007). Choice of age of experimental workers will reside solely on the purpose of the experiment, and is largely related to collection method (see sections 4.2, 4.3, 4.4). Researchers must ensure that experimental individuals are of a homogeneous age. If they are not, then heterogeneously aged individuals, or those of undefined age, should be evenly distributed among all cages.

4.1.4. Queen status of source colonies used to obtain adult workers for laboratory experiments

A queen is the typical reproductive phenotype in honey bee colonies. Not only is she responsible for egg production, but also for producing pheromones that can greatly influence worker behaviour (e.g. queen rearing) and physiology (e.g. worker ovary development) (Winston, 1987; Winston and Slessor, 1992; Slessor *et al.*, 2005). Health and age of queens are critical, as Milne (1982) observed that progeny of some queens exhibited early death in laboratory cages; this likely had a genetic component, and could be avoided when young laying queens were used. Experimental honey bees should be obtained from colonies that possess a young, mated, laying queen.

4.1.5. Strength of source colonies used to obtain adult workers for laboratory experiments

Source colonies for experimental honey bees should contain appropriate adult brood : food (i.e. honey and bee bread) ratios to ensure that workers are properly nourished, as well as adult and developing individuals of all ages, and food stores from poly-floral sources. Colonies should also be of approximately equal strength because size can influence colony defensive behaviour which can subsequently effect honey bee collection (Winston, 1987). Refer to the *BEEBOOK* papers by Delaplane *et al.* (2013) for how to estimate colony strength, Human *et al.* (2013) for estimating age of developing honey bees, and Delaplane *et al.* (2013) for estimating floral sources, as well as section 4 in this paper for obtaining workers from colonies for experiments.

4.1.6. Health of source colonies used to obtain adult workers for laboratory experiments

Multiple environmental pressures, such as pests, pathogens, and agricultural practices, acting singly or in combination, can influence honey bee health (Neumann and Carreck, 2010; vanEngelsdorp and Meixner, 2010; Williams *et al.*, 2010), and therefore potentially their response to experimental treatments. Ideally, workers used for experiments, as well as the colonies they are sourced from, should be free of pathogens, parasites, pests, and contaminants. In most cases this may not be possible, so at the very least factors potentially confounding results should be stated. Colonies with clinical symptoms of disease (e.g. chalkbrood mummies, foulbrood scales, dysentery, and individuals with deformed wings) should not be used, and infestation levels of the parasitic mite *Varroa destructor* on adults should be below economic and treatment thresholds for the particular region and time of year.

The purpose of the experiment will determine if presence/absence of certain pathogens, parasites, and pests of honey bees need to be considered. Refer to respective *BEEBOOK* papers for pathogen-specific diagnostic methods (Anderson *et al.* (2013) for *Tropilaelaps* spp., de Graaf *et al.* (2013) for American foulbrood, de Miranda *et al.* (2013) for viruses, Dietemann *et al.* (2013) for *Varroa* spp., Ellis *et al.* (2013) for wax moth, Forsgren *et al.* (2013) for European foulbrood, Fries *et al.* (2013) for *Nosema* spp., Jensen *et al.* (2013) for fungi, Neumann *et al.* (2013) for small hive beetle, and Sammataro *et al.* (2013) for tracheal mites).

4.1.7. Beekeeper management of source colonies used to obtain adult workers for laboratory experiments

Beekeeper management practices can greatly influence a honey bee colony. For example, miticides used to control *V. destructor* can be found at high levels in honey bee products (Mullin *et al.,* 2010), and could potentially be responsible for sub-lethal or synergistic effects on individuals (Alaux *et al.*, 2009; Wu *et al.*, 2011). Additionally, pathogens can occur in bee products (Gilliam, 1979), and be a local source of infection (Fries, 1993). Both chemicals residues and pathogens can accumulate on comb over time. It is important to fully understand beekeeper management of source colonies in the months, and even years, preceding collection of honey bees for laboratory tests. This includes gathering information on timing and type of medications, addition or removal of honey supers, condition of comb (e.g. old versus new), timing of previous comb replacement, queen age, requeening events, and origin of honey bee materials (e.g. wax foundation sourced locally or not, organic versus non-organic, etc.). Workers should not be collected during, or within 8 weeks of, the application of any honey bee pest or parasite control treatment. This will ensure that newly emerging workers and most "summer" individuals performing tasks inside the hive were not exposed to treatments (Winston, 1987). Researchers should acknowledge that residues from some treatments

may persist in honey bee products and colonies for an extended period (Lodesani *et al.*, 2008; Mullin *et al.* 2010).

4.1.8. Environment surrounding source colonies used to obtain adult workers for laboratory experiments

Source colonies should not be located in intensive agricultural areas with high agricultural chemical use or low bee-plant diversity because of potential sub-lethal or synergistic effects of residues (Alaux *et al.*, 2009; Wu *et al.*, 2011) and the importance of nutrition to honey bee vitality (Brodschneider and Crailsheim, 2010), respectively. Additionally, knowledge of neighbouring apiaries is useful because of the potential for disease transmission. Note that honey bee poisoning can also occur in non-agricultural areas (e.g. natural or urban areas), normally because of misuse of pesticides on attractive flowering garden plants. These toxic pesticides used during blooming may cause important honey bee loss, although their residues may not necessarily will be found in hive matrices as individuals may die before returning to the colony. These deaths can alter the age profile of workers available for collection for experiments. Therefore, one should not collect workers from colonies that experience unexpected depopulation or abnormal honey bee mortality in front of the hive. Although costly, analyses of honey bees and their products (especially bee bread) can be used to quantify chemical residues within colonies. Local information on pesticide applications may also be gleaned from agricultural pesticide-use databases when they are available.

Vegetation surveys can be performed within normal worker foraging distances from the colony – within a 2 km radius of the hive (Winston, 1987) – to identify major nectar and pollen producing plants. Careful inspection of bee bread will also determine diversity of floral sources. This can be performed by visualizing pollen grain morphology using microscopy, or more crudely by colour differentiation (see Delaplane *et al.* (2013) in the pollination paper of the *BEEBOOK* for details on identifying plant species using pollen grains).

4.1.9. Weather before and during collection of adult workers for laboratory experiments

Weather events prior to honey bee collection can have a dramatic influence on colony strength and health. Periods of dearth or drought can greatly reduce food reserves within colonies (Schmickl and Crailsheim, 2001); whereas, prolonged periods of unfavourable flying conditions (e.g. rain, snow, wind) can confine workers to colonies for extended periods, and may promote overall colony stress (Schmickl and Crailsheim, 2007) and intra-colony disease transmission (Fries, 1993).

Current weather can also greatly affect flying patterns, and therefore potentially influence worker collection. Age polyethism observed in honey bees typically dictates that older individuals perform tasks outside of the colony, such as ventilating and guarding the colony, as well as collecting food (Winston, 1987). Therefore during unfavourable conditions a high number of older individuals will be present in the colony.

Both temperature and solar radiation influence foraging patterns (Burrill and Dietz, 1981). For example, foraging activity is positively related to temperature between 12 - 20°C (below 12°C honey bees typically do not search for food). Similarly, a positive relationship between foraging and solar radiation exists at low radiation intensities (i.e. < 0.66 langley (common unit of energy distribution for measuring solar radiation); the opposite occurs at high intensities). Expectedly, higher winds and rainfall also results in decreasing foraging activity, and therefore a greater number of older individuals in the colony (Winston, 1987). Sunny, warm weather conditions are optimal for collecting workers for experiments because fewer constraints are likely to limit the ability of workers to perform their required tasks. Regardless of weather, current conditions during collection, or unusual weather events prior to collection that may influence the nature of worker collection, should always be noted.

4.1.10. Diurnal timing of collection of adult workers for laboratory experiments

Flight patterns can also be influenced by time of day, possibly because of variations in flower nectar production (Winston, 1987). Foraging peaks typically late in both the morning and the afternoon, but lulls during the early afternoon (i.e. during the high sun period), and is infrequent between dusk and dawn (i.e. during the night) (Burrill and Dietz, 1981). Periods of high foraging activity are typically suitable for collecting workers for experiments because workers are more likely to be performing their tasks normally.

4.2. Collecting newly emerged workers for laboratory experiments

4.2.1. Considerations for choosing to use newly emerged workers for laboratory experiments

Collecting newly emerged workers, or "tenerals" as described by Winston (1987), is an easy and accurate method for obtaining large quantities of adults of a homogenous age. Newly emerged adults can be an important source of relatively 'clean' individuals because they are exposed to hive and environmental conditions less than older ones. It should be noted that it is virtually impossible to prevent, with 100% certainty, horizontal residue or pathogen contamination because of conditions in which workers develop within the colony (i.e. developing individuals are fed bee products in a wax cell) and because newly emerged workers, even caged on a frame in the laboratory, will feed on frame food stores, manipulate wax, and interact with previously emerged individuals. Newly emerged workers are also appropriate to use when examining possible treatment effects on honey bee longevity, or intra-host parasite development because individuals can be maintained in the laboratory for a number of weeks.

4.2.2. Obtaining newly emerged workers for laboratory experiments without caging queens

Here is the most practical way to obtain newly emerged workers with relatively low chemical residue or pathogen exposure:

1. Choose appropriate colonies from which to collect workers from based on health, environmental, genetic, and experimental design considerations discussed in sections 2 and 4.1.
2. Select frames containing enough capped brood that will emerge in one to three days (i.e. pupae with dark eyes and cuticle) to ensure that the required number of adults can be obtained. Consult the *BEEBOOK* paper on miscellaneous methods by (Human *et al.*, 2013) for information on how to obtain brood and adults of known age. Frames should be relatively new, not appear dark in colour or be soiled with faecal material or fungi, and should have few food stores.
3. Remove all adult honey bees from the frame using a bee brush or by gently shaking the frame over the colony.
4. Place the frame in an appropriate frame cage (see sections 5.2.1 and 5.3.1) that is outfitted with food (see section 7). Frame food stores and emerging honey bees can be segregated by cutting away honey and bee bread, or by installing 0.3-cm diameter aluminium hardware cloth screen around the stored food to keep workers from feeding.
5. Transfer the frame cage to a laboratory incubator maintained at conditions discussed in section 6.
6. Monitor the frame frequently to limit exposure of newly emerged workers to the frame. Individuals should be removed from the brood frame at least every 12-24 hours to obtain age homogeneity; however, frequency of worker removal from the frame can be adjusted according to the needs of the study and to reduce contamination by pathogens and chemical residues.
7. Gently brush newly emerged individuals into appropriate hoarding cages containing appropriate food (see sections 5 and 7). Newly emerged adults can also be removed gently from cells using a forceps before full emergence to further reduce potential for contamination. These individuals can be identified by small perforations in the wax capping of the brood cells. Care must be taken because the cuticle may not be fully hardened, and individuals can be easily damaged.
8. Immediately place the hoarding cage containing newly emerged adults in a laboratory incubator maintained at conditions discussed in section 6.

4.2.3. Obtaining newly emerged workers for laboratory experiments by caging queens

Newly emerged workers can also be collected from pre-selected brood frames that queens were previously restricted onto.

To obtain newly emerged workers from a frame that the queen was caged onto:

1. Identify suitable source colonies, as discussed in section 4.1, and brood frames, as discussed by Crailsheim *et al.* (2013) in the *in vitro* larval rearing paper of the *BEEBOOK*. A frame previously used for brood production that is relatively new (i.e. not containing dark, soiled comb) and has adequate empty cells is most suitable, and will likely contain fewer pathogens and environmental contaminants. A frame from the source colony will likely be most successful for rearing known age cohorts of workers; however, one from a different colony can also be used. Number of empty cells available for egg laying will be determined by the number of individuals needed for experiments. Brood mortality of approximately 20% should be expected (Fukuda and Sakagami, 1986).
2. Locate the queen in the source colony and gently place her on the chosen brood frame by grasping her wings. A clip queen catcher cage can also be used to move her. Refer to the *BEEBOOK* paper by Human *et al.* (2013) for handling honey bees. Ensure that at least a few hundred workers are on the frame before the queen is moved. These workers can either be ones that were on the frame originally or ones brushed from another brood frame in the same colony that contains open brood. This will serve to calm her and will lessen the chances that she runs or flies, or is crushed during caging.
3. Carefully place the frame, containing the queen and workers, in a queen excluder cage (Fig. 1), and seal it, ensuring the queen is not crushed. See section 5.2.1 for discussions on minimizing pathogen and environmental contaminant exposure when using cages.

Fig. 1. A brood frame containing workers, the queen, and many empty cells is being inserted into a queen excluder cage. Slits between 4.3 and 4.4 mm wide allow worker movement to and from the frame, but restrict queen passage.

Fig. 2. A frame caged in a queen excluder placed in the middle of the brood nest, between frames containing eggs and larvae.

4. Place the caged frame in the broodnest, preferably between two brood frames containing eggs and larvae (Fig. 2). This will improve chances that the newly-laid eggs are accepted by the colony. Refer to Human *et al.* (2013) in the miscellaneous methods paper of the *BEEBOOK* for estimating developing worker bee age.
5. After a defined period of time, remove the frame from the queen excluder cage and place it, with brood and the queen, back into the colony in its previous position. Mark the frame with a permanent marker or a coloured drawing pin to help locate it in the future. The number of honey bees required for experiments will determine the length of time the queen is confined to the frame. Queens typically lay between 5-35 eggs per hour (Allen, 1960), and frames can be checked every 24 hours to determine if enough eggs have been laid by inspecting cells through the queen excluder cage with the aid of a flashlight. Refer to the miscellaneous methods paper of the *BEEBOOK* by Human *et al.* (2013) for identifying eggs. It is possible that the queen will not begin egg laying until a few hours after initial isolation. Queens should not be confined to the frame for more than 72 hours, or when the availability of cells for egg laying is low, to avoid significant disruption of brood rearing in the colony. Homogeneity of age of newly emerged bees will also determine the length the queen is restricted to the frame, although this can also be controlled for during regular removal of newly emerged adults from the frame.
6. Remove the frame 19-20 days after initial queen restriction, just prior to adult emergence (Winston, 1987). The frames can be removed later if egg laying was significantly delayed, but care must be taken to prevent workers from emerging in the colony. Although a worker will usually emerge from a cell 21 days after an egg was laid, development time can vary between 20-28 days depending on environmental conditions such as temperature and nutrition (Winston, 1987).
7. The frame and newly emerged adults can be subsequently handled according to #5, 6, and 7 of section 4.2.2.

4.2.4. Obtaining newly emerged workers for laboratory experiments by *in vitro* rearing

Newly emerged workers can also be obtained for experiments using *in vitro* rearing techniques described by Crailsheim *et al.* (2013) in the *in vitro* rearing paper of the *BEEBOOK*. This option is particularly useful to study experimental treatment effects in adults exposed during development.

4.3. Collecting adult workers of an undefined age for laboratory experiments

4.3.1. Considerations for choosing to use adult workers of an undefined age for laboratory experiments

Under certain circumstances it is not necessary to collect individuals of a known age. Although there is a tendency due to age polyethism (i.e. temporal division of labour) for young and old workers to be found in the centre or periphery of the broodnest, respectively (Seeley, 1982), or for older workers to perform jobs outside of the hive (Winston, 1987), distribution of age cohorts throughout the colony is dynamic and can be influenced by colonial needs (Calderone, 1995; van der Steen *et al.,* 2012). See Human *et al.* (2013) for a summary of worker development. We describe here how to sample workers of an undefined age. Under the appropriate conditions (see sections 4.1.9 and 4.1.10) broad functional groups of workers can be collected (e.g. individuals performing tasks in the hive versus those performing tasks outside the hive).

4.3.2. Challenges associated with collecting adult workers of an undefined age for laboratory experiments

Obtaining workers of an undefined age for an experiment usually requires the collector to physically open the colony or stand immediately in front of it to retrieve individuals. Collecting flying workers at the colony entrance can particularly agitate colonies, and may initiate a defensive response that will result in a mass exodus of guards from the hive (Breed *et al.*, 2004). Thus, agitation of colonies should be minimized because it can influence worker collection.

4.3.3. Collecting flying adult workers of an undefined age for laboratory experiments

Workers performing tasks outside of the hive are generally older than individuals working within (Winston, 1987), but as discussed in section 4.3.1., collecting workers of a particular age, or even performing a specific task, may not be straightforward. Returning pollen foragers can easily be observed by presence of corbicular pollen on their hind legs (Fig. 3).

Fig. 3. A foraging worker honey bee with corbicular pollen (black arrow) on its hind leg.

Fig. 4. Collecting exiting worker honey bees using a clear container with mesh bottom from a colony with a reduced entrance size.

It may be helpful to reduce the size of the hive entrance when performing certain collection methods to limit the area individuals may pass in or out of the colony. Completely sealing the hive for short periods (i.e. < 30 minutes) can also be used to collect returning flying individuals as they accumulate on the landing board. Time required to collect an appropriate number of flying workers can be estimated by observing the hive entrance for 2 - 3 minutes. Most foragers perform approximately 10 - 15 trips per day (Winston, 1987); however, length of collection time will be influenced by time of day and weather (as discussed in sections 4.1.9 and 4.1.10), as well as size of colony.

4.3.3.1. Collecting flying adult workers of an undefined age for laboratory experiments using a forceps

Exiting workers can be collected individually using forceps.

1. Stand beside, and not in front of, the colony.
2. During normal flight activity, grasp appropriate individuals by a leg or wing using forceps. Care must be taken that individuals are not damaged during collection. Refer to Human *et al.* (2013) in the miscellaneous methods paper of the *BEEBOOK* for details on handling honey bees using forceps.
3. Place collected workers in a ventilated hoarding cage with appropriate food (see sections 5 and 7).
4. Immediately transfer the hoarding cage to a laboratory incubator maintained at conditions discussed in section 6.

4.3.3.2. Collecting flying adult workers of an undefined age for laboratory experiments using a container

Workers leaving the hive can also be collected using a clear, wide-mouthed, well ventilated transparent container (with associated lid) as they depart the hive entrance (Fig. 4). Ventilation can be provided by perforating the container with numerous 2 mm-sized holes or by replacing a large portion of the base of the container with a mesh screen. Efficiency of this method depends on flying patterns of the colony, the ease of attaching the lid to the container, and the reflexes of the collector. Alternatively, a UV light-permeable plexiglass pyramid (height = 30 cm, apex 3,5 x 3, 5 cm, base 18 x 18 cm) that is closable at the apex and the base can be placed tightly around the hive entrance to prevent exiting foragers from escaping (e.g. Felsenberg, 2011; Matsumoto *et al.*, 2012).

1. Stand beside the colony and hold a wide-mouthed clear container immediately against the front of the colony so that exiting individuals will fly or walk into the container. It may be helpful to reduce the size of the hive entrance to funnel greater numbers of exiting workers directly into the container and to use a container with a rectangular shaped opening that fits better to the flight board and hive entrance.
2. Seal the container when an appropriate quantity of workers is collected.
3. Shake the collected individuals gently into a ventilated hoarding cage containing food (described in sections 5 and 7).
4. Transfer the hoarding cage to a laboratory incubator maintained at conditions discussed in section 6.

4.3.3.3. Collecting flying adult workers of an undefined age for laboratory experiments using an entrance trap

Entrance traps allow for a large number of exiting workers to be collected from colonies with minimal disturbance because workers will eventually not view the trap as a foreign object. The Bologna Trap has a particularly effective design (Medrzycki, 2013).

4.3.3.3.1. Bologna Trap description for collecting adult workers for laboratory experiments

The Bologna Trap acts as a funnel that can be placed over the lower front portion of a hive. Because the trap can remain on the colony for an indefinite period of time in an open position, workers will pass in and out of the colony normally (Fig. 5). The bottom of the funnel acts as an extension of the flight board, sealing tightly to it and to the front of the hive so that exiting individuals leave the hive and enter the trap by walking (Fig. 6). The funnel is curved upwards, reaching an

Fig. 5. Bologna Traps, without collection containers, attached to the entrance of honey bee colonies.

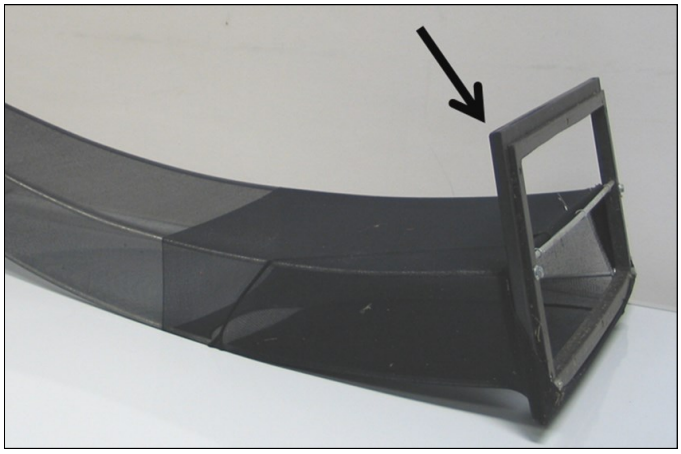

Fig. 6. A detached Bologna Trap. Arrow points to proximal portion of the trap that can be attached to the lower front portion of the hive to completely and securely surround the hive entrance.

Fig. 7. Terminal end of the Bologna Trap. The ring (*i.e.*, a lid with a large hole cut away) accommodates a collection container that can be attached to obtaining flying honey bees exiting the colony.

inclination of approximately 30°; any greater inclination may result in dead honey bees accumulating in the trap. The funnel ends with an adaptor (i.e. a lid with a large hole cut out) for where a collection container to be attached (Fig. 7). See section 4.3.3.2 for a description of a collection container.

4.3.3.3.2. Collecting flying adult workers of an undefined age for laboratory experiments using the Bologna Trap

1. Fix the trap, without the collection container, to the hive for at least 5 days before collecting workers to accustom the colony to the device. Acceptance of the trap can be verified when undertaker workers remove dead individuals from the trap. The trap can remain installed on the hive for the entire season, apart from when cleaning and repairs are required.
2. When experimental workers are needed, observe the hive entrance for 2-3 minutes, noting the number of exiting workers, to estimate approximate length of time collection is needed.
3. Install the collection container to the distal end of the funnel (Fig. 8).
4. When the appropriate number of flying workers are collected (Fig. 9), remove the collection container quickly and seal it (Fig.10).
5. Transfer collected workers by gently shaking the collection container over an open hoarding cage containing food (as discussed in sections 5 and 7)
6. Transfer the hoarding cage to a laboratory incubator maintained at conditions discussed in section 6.

4.3.4. Collecting intra-hive adult workers of an undefined age for laboratory experiments

Workers can be easily collected from frames within the colony. Because of the dynamic nature of honey bee age polyethism (Calderone, 1995; van der Steen *et al.*, 2012), it is not possible to accurately collect individuals of known ages based on location within the colony. For example, van der Steen *et al.* (2012) observed no difference in worker age classes among frames in a colony, and that approximately 60% of workers on frames were one or two weeks old.

1. Inspect the frame from which workers are to be collected from for the queen. If present, gently move her to an adjacent frame.
2. Gently brush individuals into a suitable hoarding cage (see section 5) placed below the frame using a beekeeping brush or similar tool with soft bristles. Alternatively, the frame can be gently shaken over a suitably sized open-mouthed container prior to transferring collected workers to a suitable hoarding cage.

Fig. 8. A Bologna Trap with a ventilated collection container installed on the foreground colony to obtain exiting honey bees.

Fig. 9. A ventilated collection container obtaining flying workers exiting the hive. Note that the bottom of the container is replaced with a fine mesh that is held in place using an elastic.

Fig. 10. Removing the collection container filled with exiting honey bees from the Bologna Trap.

3. Gently shake the opened hoarding cage or container for ~ 1 minute to prevent young workers from escaping by walking and to allow older flying workers to exit.
4. Close hoarding cage, or transfer remaining workers into a suitable hoarding cage with food (sections 5 and 7).
5. Immediately transfer the hoarding cage to a laboratory incubator maintained at conditions discussed in section 6.

4.4. Recommendations for choosing and collecting adult workers for laboratory experiments

The choice of type of honey bees to use during experiments, as well as when and how to collect them, is intimately tied to the hypothesis being tested. At a minimum, all possible characteristics of the experimental individuals (e.g. age), source colonies, (e.g. strength, health, subspecies), surroundings (e.g. availability of multiple nectar and pollen sources), as well as conditions during collection (e.g. time of day and year, weather conditions) and collection method (e.g. brushing from a brood frame versus collecting exiting flying workers using a hive entrance trap), should be described in detail in the methods section of each publication. Importantly, researchers must ensure that all treatments contain experimental honey bees were handled identically. The easiest approach to guarantee this is to mix honey bees from all sources evenly among all experimental cages, as suggested in this paper in section 2.4. Additional information on choosing source colonies is provided by Pirk *et al.* (2013) in the statistics paper of the *BEEBOOK*.

5. Cages in which to maintain adult workers in the laboratory

5.1. Types of cages in which to maintain adult workers in the laboratory

Generally, three types of cage design exist for maintaining adult worker honey bees outside of a colony in a laboratory:

a) caged on a frame (i.e. using a frame cage)
b) caged off a frame in a group (i.e. using a hoarding cage)
c) caged off a frame individually (i.e. using an isolation cage)

Even within these types numerous variants exist (Fig.11). Yet, despite the diversity of cage designs, very little work has investigated the influence of these differences on results of experiments using honey bees.

5.2. Choosing a suitable cage to maintain adult workers in the laboratory

5.2.1. Minimum criteria for frame and hoarding cages in which to maintain adult workers in the laboratory

Generally, frame and hoarding cages of all types should meet the following minimum criteria; however, discretion may be used

Fig. 11. The diverse assemblage of cages used for honey bee research brought by those attending a COLOSS workshop in November 2011 in Bologna, Italy.

depending on the purpose of containing honey bees (e.g. for caging newly-emerged adults in a brood frame or for performing experiments using hoarding cages).

- Cages should be used once and discarded, or sterilised and cleaned if used multiple times, to minimise contamination by pathogens and chemical residues.
- Single-use cages are recommended for studies involving pesticide toxicology because of the difficulty in removing chemical residues.
- Multiple-use cages can be used for honey-bee pathogen studies and should be made from materials that are easily sterilised (e.g. autoclaved or irradiated), such as stainless steel and glass. Type of sterilisation required will depend on the nature of the study. For example, exposure to 121°C for 30 minutes will destroy *N. ceranae* spores (Fenoy *et al.*, 2009). Metal and plastic cages can be further decontaminated using acetone*:

1. Wash cages using a standard laboratory dish washer
2. Apply a sparse quantity of technical grade 100% acetone (the preferred solvent in toxicology laboratories) to a cloth and wipe cage clean. Attention should be paid to effects of acetone on plastic cages.
3. Soak a new cloth in warm soapy water and wash/rinse cage.
4. Rinse cage with water.
5. Dry cage using a new cloth, and air-dry until all liquid evaporates.

*Refer to your own laboratory safety manual to learn how to properly work with acetone.

- Materials used to make cages should be inexpensive, and easily accessible and manipulated. Plastic and wood allow for easy modification of cages when, for example, an additional feeding device is needed.
- Cages should have a sufficient quantity of air holes to provide ventilation.
- To reduce risk of contamination by pathogens and chemical residues among cages maintained in the same incubator, ventilation holes should be covered by filter paper or similar breathable material. If vents are unfiltered, cages should face in opposite directions and should be placed sufficiently far apart to prevent inter-cage trophallaxis or frass movement.
- Cages should allow both living and dead honey bees to be easily removed during the experiment, and should prevent live bees from accidentally escaping.
- At least a portion of the cage should be transparent to allow honey bees to be observed.
- Cage size will depend on the number of honey bees to be detained. For example, 500 cm^3 (i.e. 500 ml) can easily accommodate several hundred workers, whereas cages of 100 cm^3 are suitable for maintaining 30 workers. Generally, a ratio of ~3:1 (cm^3/bee) is appropriate for maintaining less than a few hundred workers.

5.2.2. Supplementary frame and hoarding cage materials to be used when maintaining adult workers in the laboratory

Additional materials, such as comb or wax foundation (e.g. Czekońska, 2007) and plastic devices for releasing queen mandibular pheromone (QMP) (e.g. Alaux *et al.*, 2010), are sometimes used to provide more realistic conditions to honey bees. For the former, comb and wax foundation should be used with caution because both can contain chemical residues (Mullin *et al.*, 2010) and pathogens (Melathopoulos *et al.*, 2004); however, organic wax foundation is available. For the latter, QMP, composed of 5 compounds ((E)-9-oxodec-2-enoic acid (9-ODA), both enantiomers of 9-hydroxydec-2-enoic acid (9-HDA), methyl p-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA)) (Slessor *et al.*, 1988), likely promotes honey bee health and reduces stress, as well as influences brain development (Morgan *et al.*, 1998), resistance to starvation (Fischer and Grozinger, 2008), age-related division of labour (Pankiw *et al.*, 1998), and worker ovary activation (Hoover *et al.*, 2003). More studies are needed to fully understand effects of QMP on caged honey bees before it can be recommended as a regular requirement for maintaining adults in the laboratory.

5.2.3. Minimum criteria for isolation cages in which to maintain adult workers in the laboratory

In contrast to frame and hoarding cages, isolation cages are rarely used outside of studies investigating behaviour or learning. Many of the principles discussed above for frame and hoarding cages also apply to isolation cages, such as the importance of providing a sterile, well-ventilated cage.

5.3. Suitable cages in which to maintain adult workers in the laboratory

The following cage descriptions are provided by the authors to give examples of those generally meeting minimal criteria listed above. There are no doubt other cages described in detail elsewhere that are equally suitable (e.g. hoarding cages: Pernal and Currie, 2000; Evans *et al.*, 2009).

5.3.1. Example of a frame cage in which to maintain adult workers in the laboratory

Generally, a frame cage allows for a single frame to be suspended within it, and contains one or two ventilated sides that can be slid away to allow access to the frame (Fig. 12).

Fig. 12. A frame cage containing a Zander-sized frame and composed of a wooden casing, a metal screen, a glass removable sliding side, and two feeding devices. Cage courtesy of the Swiss Bee Research Centre.

5.3.2. Examples of hoarding cages in which to maintain adult workers in the laboratory

Classic hoarding cages are shaped similar to frame cages, and also contain one or two sides that may be removed (Fig. 13), although other designs exist that are cup-shaped (Fig. 14) or are modifications of the classic design with the cage rested on its side so that the top is removable (Figs. 15 and 16).

5.3.3. Examples of isolation cages in which to maintain adult workers in the laboratory

For isolation cages, modified straws with pins placed at either end, 1.5 ml microcentrifuge tubes with breathing holes drilled through the tip (Fig. 17), or 0.8 cm wide plastic Eppendorf tubes cut in half longitudinally with sticky tape restraining harnesses (Fig. 18), can be used. To our knowledge, researchers do not maintain individuals in these types of cages for more than one week. Future studies should investigate effects of isolation cages on survival and health of caged honey bees, as well as work to develop an appropriate method for maintaining individuals in isolation cages for an extended period of

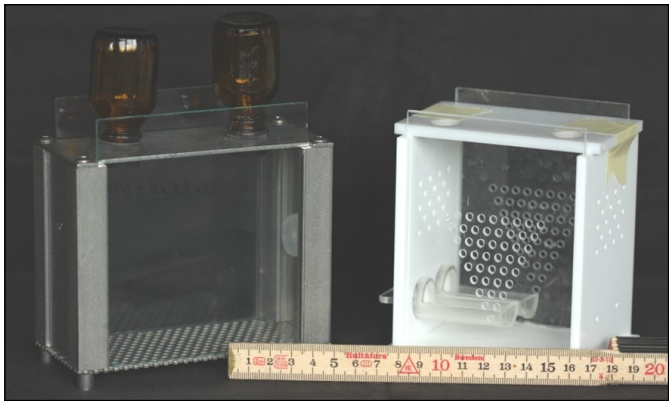

Fig. 13. Examples of 'classic' hoarding cages equipped with transparent and removable sides, ventilation holes, and multiple inputs for feeding devices. Cages courtesy of the Swiss Bee Research Centre (left) and INRA (right).

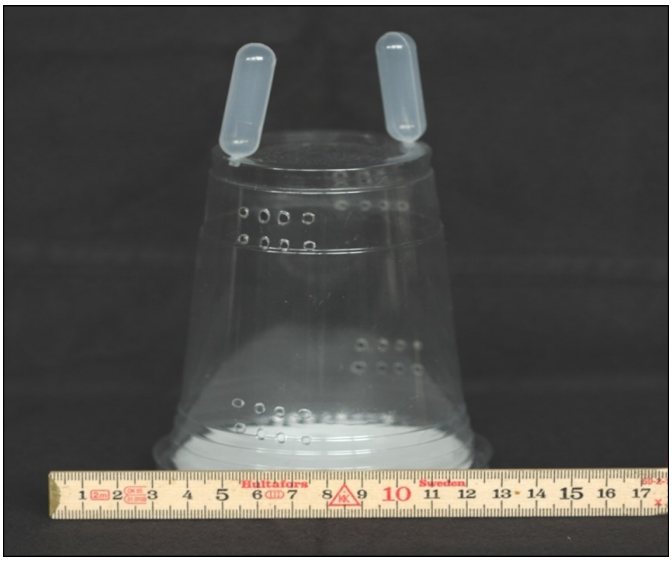

Fig. 14. Cup-shaped hoarding cage with removable base, multiple ventilation holes, and two feeding devices. Modified from Evans *et al.* (2009). Cage courtesy of ScientificBeekeeping.com

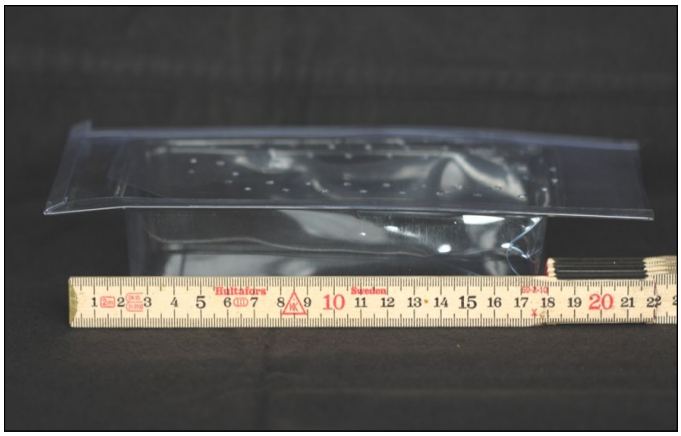

Fig. 15. Hoarding cage containing removable top, and multiple ventilation holes and feeding device inputs. Cage courtesy of Szent István University.

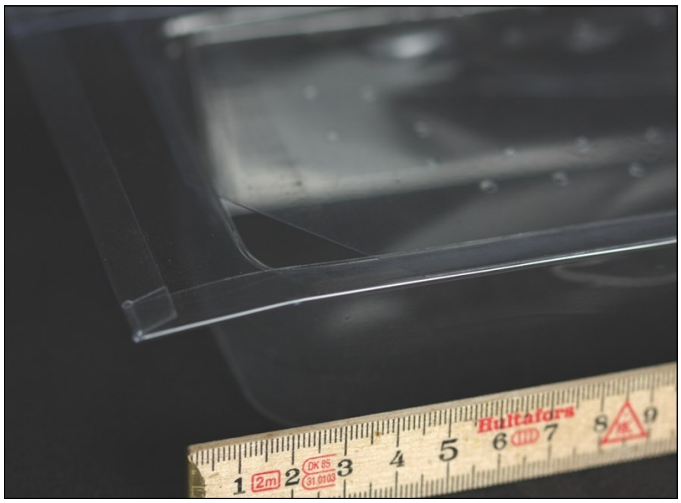

Fig. 16. Magnification of the sliding, removable top of the cage presented in *Fig. 15.* Note the removed corner to facilitate addition or removal of honey bees. Cage courtesy of Szent István University.

Fig. 17. Isolation cages created by drilling 2-3 mm ventilation holed in the tip of 1.5-ml microcentrifuge tubes. Cages and storing device courtesy of Ulrike Hartmann, Swiss Bee Research Centre.

Fig. 18. Isolation cage constructed using a plastic Eppendorf tube cut in half longitudinally and sticky tape harnesses. Tube height, and outer and inner diameters = 3, 1, and 0.8 cm, respectively. Cages courtesy of CWW Pirk. Photo: V Dietemann

time. This could potentially greatly increase experiment sample size compared to hoarding cages that must include cages, rather than individuals, as number of replicates.

6. Incubator conditions

6.1. Regulation of biophysical properties within colonies

Honey bees are renowned for cooperatively maintaining nest homeostasis by regulating biophysical properties such as temperature, humidity, and respiratory gases within a colony. In doing so, they create a suitable environment that moderates adverse conditions (Danks, 2002). When maintained in an incubator, however, appropriate conditions must be provided, regardless of if honey bees are kept individually or in small groups. Because biophysical properties, whether in a colony or an incubator, are intimately connected to water loss, researchers must consider not only chamber conditions, but also water availability (i.e. both drinking and vapour water), when maintaining adults in the laboratory. Although honey bees are relatively tolerant to changes in thermal and moisture conditions, it is recommended that adult honey bees be maintained in conditions as close to their natural environment as possible.

6.2. Temperature

6.2.1. Honey bee intra-hive temperature requirements

Despite considerable changes in ambient air temperature, honey bees typically maintain their brood nest between 32 and 36°C by adjusting their metabolism and by using a number of behavioural methods (Stabentheiner *et al.*, 2010) to ensure optimal brood development. Nevertheless, outer edges of honey bee clusters can drop to as low as 10°C in winter when no brood is present (Seeley, 2010). Most laboratory studies maintained caged honey bees between 25-34°C (e.g. Webster, 1994; Higes *et al.,* 2007; Paxton *et al.*, 2007; Alaux *et al.*, 2009), and 25 ± 2°C is recommended for testing acute oral toxicity of chemicals (OECD, 1998).

6.2.2. Recommendations for incubator temperature for maintaining adult workers in the laboratory

Frames of brood should be maintained at 34.5°C for optimal brood development (Heran, 1952; Crailsheim *et al.*, 2012); whereas, we recommend keeping adults at 30°C, based on optimal respiration at 32°C (Allen, 1959) and honey bee thermal preference of 28°C (Schmolz *et al.*, 2002). Generally these recommendations are also appropriate when performing experiments; however, further adjustments to temperature may be required. For example, a recent study investigating acute oral toxicity of chemicals to honey bees under laboratory conditions suggested that these evaluations should be performed at both 25 and 35°C to account for the wide range of temperatures to

which honey bees are exposed (Medrzycki and Tosi, 2012). When obtaining newly-emerged honey bees from a brood frame maintained at 34.5°C in an incubator, young honey bees (i.e. individuals 0 to 24 hours old) should be transferred from a caged frame containing brood to one or more cages that are maintained at 30°C. More details on obtaining newly emerged honey bees from brood comb are provided in section 4.2.

6.3. Relative humidity

6.3.1. Honey bee intra-hive relative humidity requirements

Humidity within a colony can also be influenced by honey bees, albeit to a lesser extent than temperature (Human *et al.*, 2006). Similar to temperature, relative humidity can differ among areas of a colony (Human *et al.*, 2006), but also fluctuate substantially because of breathing events that exchange stale air at optimal humidity with air at ambient humidity (Southwick and Moritz, 1987). Relative humidity within honey bee colonies (among frames and not within capped brood cells) is typically between 50 and 80% (Human *et al.*, 2006; V. Dietemann, pers. comm.), and when given a choice between a range of relative humidities (i.e. 24, 40, 55, 75, and 90%), honey bees showed a preference for 75% (Ellis *et al.*, 2008). The OECD (1998) recommends relative humidity to be between 50-70% for laboratory testing of acute oral toxicity of chemicals.

6.3.2. Regulating incubator relative humidity for maintaining adult workers in the laboratory

If the laboratory is not equipped with an incubator capable of automatically regulating a desired relative humidity, then it can be attained easily using two methods. One can also refer to methods discussed in the *in vitro* rearing paper of the *BEEBOOK* Crailsheim *et al.* (2013) for appropriate relative humidity conditions for maintaining brood in the laboratory.

6.3.2.1. Regulating incubator relative humidity for maintaining adult workers in the laboratory using an open water basin

Relative humidity can be regulated by placing open containers filled with water at the bottom of the incubator (Fig. 19). In some cases, a suitably hung cloth wick can be used to promote evaporation.

6.3.2.2. Regulating incubator relative humidity for maintaining adult workers in the laboratory using a saturated salt solution

If an open basin of water cannot maintain the incubator at the desired condition, then further regulation can be provided using saturated salt solutions. Use of these salts is summarized here, but discussed in further detail by Wexler and Brombacker (1951) and Winston and Bates (1960).

Fig. 19. Regulation of incubator relative humidity using an open water basin.

6.3.2.2.1. Criteria for using saturated salts to regulate incubator relative humidity for maintaining adult workers in the laboratory

The following criteria are discussed by Winston and Bates (1960). Expected relative humidity values produced using saturated salt solutions may vary when experimental conditions do not permit all criteria to be met.

1. Container (i.e. incubator) must be a closed system.
2. A fan to distribute air should be provided when incubator volume is >1 litre.
3. Surface area of the solution should be as large as possible.
4. Reagent grade chemicals should be used to allow for reproducibility.

6.3.2.2.2. Choosing appropriate saturated salts for regulating incubator relative humidity for maintaining adult workers in the laboratory

A variety of salts can produce a wide range of relative humidities at many defined temperatures (see Table 1 in Winston and Bates (1960)). Choice of these salts should be determined by desired relative humidity and temperature conditions. Sodium chloride (NaCl) is easily available and can maintain relative humidity at ~75% over various temperatures when certain criteria are met (section 6.3.2.2.1). Sodium chloride can still be used despite circumstances when all criteria are not met, especially when an open basin of water alone cannot regulate the desired conditions; however, constant vigilance of relative humidity is required (section 6.3.3).

6.3.2.2.3. Preparing a saturated salt solution for regulating incubator relative humidity for maintaining adult workers in the laboratory

The following is an example of how to create approximately one litre of sodium chloride saturated salt solution:

1. Heat one litre water slowly in a two litre glass beaker.
2. Place beaker on standard laboratory magnetic stirrer.
3. During heating, gradually add ~400 g sodium chloride to water until crystals do not dissolve any further; this will slightly increase the volume of the solution.
4. Mix solution using stirrer.
5. Continue adding sodium chloride until a gentle boil is reached and no further salt will dissolve.
6. Remove solution from heat, pour in appropriate, open-mouthed basin, and let cool before transferring to the incubator. Solution should contain a mixture of crystals and liquid.
7. Use salt solution for multiple weeks; replace when no water is present or when fungi or bacterial growth occurs.

6.3.3. Monitoring and recording incubator relative humidity when maintaining adult workers in the laboratory

Small changes in ambient weather, as well as the opening of the incubator door, can significantly affect incubator relative humidity, especially when the total volume of the chamber is greater than one litre (Rockland, 1960; Winston and Bates, 1960). Because of this, an accurate, reliable data recorder or a digital measuring device should be used to document relative humidity, as well as temperature, over time. Numerous types of equipment are available, such as the iButton (Maxim Integrated Products, San Jose, United States) or HOBO (Onset Computer Corporation, Cape Cod, United States).

6.3.4. Recommendations for incubator relative humidity for maintaining adult workers in the laboratory

Considering natural colony conditions and worker preference, we recommend that adult workers of all ages should be maintained at 60-70% relative humidity in the laboratory.

Pre-trials will be needed to determine water surface area, frequency of water replacement, and choice of salt needed to sustain appropriate levels because incubator size and air exchange with the ambient surroundings will greatly influence relative humidity.

6.4. Light

6.4.1. Natural honey bee light conditions

Honey bees typically spend a considerable amount of their lives in mostly dark conditions within the hive, although late in life, light-dark cycles play a crucial role in determining foraging rhythm of workers (Moore, 2001). An exception includes some *Apis mellifera adansonii* that nest in the open (Fletcher, 1978). Despite phototaxis (i.e. movement toward or away from a light stimulus) varying relative to bee age, light intensity, and light wavelength (Menzel and Greggers, 1985; Ben-Shahari *et al.*, 2003; Erber *et al.*, 2006), permanent exposure to honey bee-visible light can affect hoarding behaviour (i.e. the collection and storage of food in the honey stomach) of caged honey bees (Free and Williams, 1972). To our knowledge, honey bees in the laboratory are always maintained in complete darkness (e.g. Malone and Stefanovic, 1999; Maistrello *et al.,* 2008; Alaux *et al.*, 2009); however, many studies fail to report light conditions.

6.4.2. Recommendations for incubator light conditions for maintaining adult workers in the laboratory

Caged workers should be maintained in an incubator under dark conditions. Workers and cages should be examined and manipulated under dim light conditions, preferably using red light that emits 660-670 nm wavelengths that are not visible to honey bees (Menzel and Backhaus, 1991). To produce light of this wavelength, special bulbs can be purchased or standard incandescent bulbs emitting human-visible light can be covered with a red lens so that light produced is of the appropriate wavelength.

6.5. Ventilation

6.5.1. Honey bee ventilation requirements

Honey bees rely on a permanent supply of oxygen to survive. Because carbon dioxide within colonies can reach levels much higher than normal atmospheric levels (0.04%) (Nicolas and Sillans, 1989), honey bees use fanning and gas exchange events to expel carbon dioxide rich air (Southwick and Moritz, 1987; Nicolas and Sillans, 1989) to maintain levels between 0.1 - 4.3% (Seeley, 1974). Carbon dioxide can also reach high levels within cages and incubators that do not provide adequate air exchange and ventilation with ambient fresh air.

6.5.2. Recommendations for incubator ventilation with ambient air for maintaining adult workers in the laboratory

It is extremely important that cages allow for appropriate ventilation, and that incubators are equipped with air exchangers or passive vents at a minimum. Although air exchange occurs every time an incubator is opened, this technique should not be relied upon because air exchange should be permanent and opening the chamber regularly will disturb caged workers. To minimise effects of potential differences in gas composition within an incubator on experimental honey bees, cages of each treatment group should be homogenously distributed in the useable space of an incubator and a small fan should be used to promote air homogenisation. More information on effects of carbon dioxide on honey bees can be found in the *BEEBOOK* paper on miscellaneous methods (Human *et al.*, 2013).

7. Nutrition

7.1. Nutritional requirements of worker honey bees

Diet can affect honey bees in numerous ways including, for example, longevity (Schmidt *et al.*, 1987) and physiology (Alaux *et al.*, 2010). Under natural conditions, honey bees receive carbohydrates and proteins they require by consuming nectar and pollen stored in a colony as honey and bee bread, respectively. Carbohydrates are the source of energy for workers; whereas, proteins are crucial for building and maintaining tissues (e.g. Hersch *et al.*, 1978; Pernal and Currie, 2000). Additional nutrients, such as vitamins, minerals, and lipids, are also obtained from pollen, although their importance are not well understood (Brodschneider and Crailsheim, 2010).

For proper growth and maintenance, each worker larva requires 59.4 mg of carbohydrates and 5.4 mg of pollen during their development (Rortais *et al.*, 2005); whereas adult workers require ~4 mg of utilizable sugars (Barker and Lehner, 1974) and consume ~5 mg pollen (Pernal & Currie 2000) per day. Interestingly, under laboratory conditions caged workers self regulated their intake at approximately 10% proteins and 90% carbohydrates (Altaye *et al.*, 2010). Although providing laboratory workers with these natural food types may not always be practical, or even ideal, it is necessary that they receive in some form appropriate quantities of essential nutrients that provide energy and promote proper growth and development (e.g. Pernal and Currie, 2000; Brodschneider and Crailsheim, 2010).

7.2. Carbohydrates

7.2.1. Types of carbohydrates to provide to caged adult workers in the laboratory

Honey bees are capable of surviving long periods on carbohydrates alone, although median lethal time (LT_{50}) can vary significantly by substrate (i.e. LT_{50} = 56.3, 37.7, and 31.3 days, respectively, for sucrose, high-fructose corn syrup, and honey) (Barker and Lehner, 1978). Additionally, recent data suggest type of carbohydrate can influence detoxification in honey bees (Johnson *et al.*, 2012), further underlining the importance of carefully choosing source of carbohydrate to feed to workers.

7.2.1.1. Providing honey to caged adult workers in the laboratory

Honey is the natural carbohydrate source of honey bees, and can be easily collected from a colony; however, it is difficult to standardize given variation in composition due to floral diversity (e.g. White and Doner, 1980). Additionally, it may contain chemical residues (Chauzat *et al.,* 2009) and microflora (Gilliam, 1997), including pathogens (Bakonyi *et al.,* 2003), despite its antibacterial properties (Kwakman *et al.*, 2010). Honey can be collected from honey supers and provided pure, diluted 1:1 (volume/volume) with tap water, or as a paste consisting of 70% (volume/volume) powdered sucrose and 30% pure honey (e.g. Alaux *et al.*, 2011a). Refer to section 7.5 in this paper for a discussion on providing water to caged honey bees in the laboratory.

7.2.1.2. Providing sucrose solution to caged adult workers in the laboratory

Sucrose solutions can sustain workers for long periods of time in the laboratory (Barker and Lehner, 1978), and they are frequently used (e.g. Malone and Stefanovic, 1999; Paxton *et al.*, 2007; Forsgren and Fries, 2010). Solutions can be made simply by dissolving sucrose sugar in water. The sucrose should be white refined table sugar intended for human consumption that can be purchased in a supermarket.

To make a 100 ml volume 50% (weight/volume) solution, for example:

1. Add 50 g table sugar (sucrose) to a 200 ml glass beaker.
2. Add tap water until total volume reaches 100 ml.
3. Stir until all sugar is dissolved (i.e. < 5 mins.). If needed, water can be briefly warmed to < 50°C to help dissolve the sugar, but it should be cooled to room temperature before it is provided to caged workers.
4. Provide immediately to caged workers.
5. Store surplus solution for no more than 2-3 days at 4°C. Prior to feeding, remove solution from fridge a few hours before providing it to caged workers in order to prevent feeder leakage caused by the solution warming.

7.2.1.3. Providing sucrose paste to caged adult workers in the laboratory

Although it is used less frequently during laboratory assays compared to sucrose solutions (e.g. Maistrello *et al.,* 2008; Alaux *et al.*, 2009), sucrose paste is often provided to queens and accompanying nurses that are maintained in cages in a laboratory. Because it is a solid, the paste should be provided using devices designed for protein distribution, as explained in section 7.3.2. Water should also be given in a separate feeder when sucrose paste is the sole source of carbohydrates; refer to section 7.5. for details on providing water to caged workers.

To make 100 g of 95% (weight/weight) sucrose candy, for example:

1. Add 95 g powdered sucrose sugar to a 200 ml glass beaker.
2. Add 5 g tap water to the beaker.
3. Stir until a paste is created. Consistency should be similar to soft dough, and it should not ooze.

7.2.2. Feeding devices for providing carbohydrates to caged adult workers in the laboratory

Numerous types of devices can be used to provide liquid carbohydrates to caged honey bees. Feeding devices must fulfil the following minimum criteria:

- Allows workers to drink safely, without drowning.
- Holds the respective volume securely, minimises evaporation, and prevents leakage; a small piece of paper tissue can be inserted in the feeder over top of the feeding site to prevent leakage.

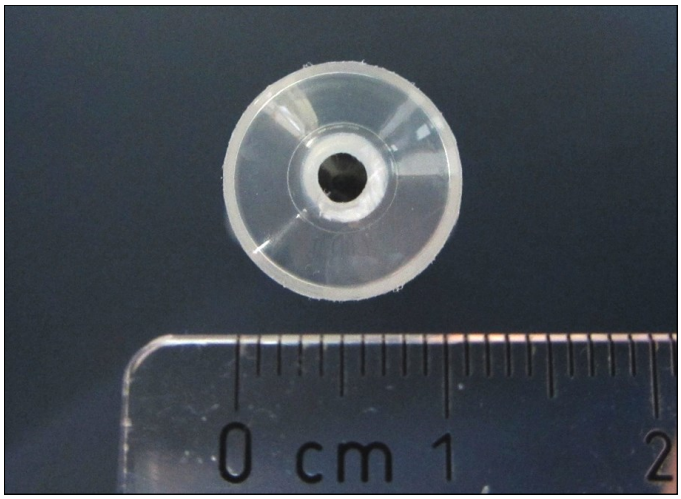

Fig. 20. A disposable 5 ml plastic syringe with Luer connection fitting removed to create a 2 mm hole revealing the black plunger.

- Ensures feeding sites are not easily blocked by crystallisation; size of feeding site hole that is dispensing food, as well as water concentration of carbohydrate, will influence crystallisation. Since no data are currently available on the subject, pre-trials will determine an appropriate size of feeding site.
- Allows for quick and easy replenishment of the solution, as well as measurement of consumption, that minimises accidental escape of experimental individuals and preferably does not require opening cages.

For workers in frame or hoarding cages (refer to section 5), a simple disposable feeding device can be made using a microcentrifuge tube (< 2 ml) with two to three small holes 1-2 mm wide drilled into the bottom or by using a syringe with the needle removed and adaptor cut away to reveal a 2-5 mm wide hole (Fig. 20). Alternatively, a feeding device can be created by drilling a single 2-5 mm wide hole in the base, as well as two 2-5 ml sized holes on the sides ~5 mm from the tip to prevent air bubbles from forming at the bottom; a small piece of tissue paper can be inserted into the tip to prevent leakage. Gravity feeders, created by inverting a jar with a lid containing a single large hole (i.e. 5 mm) screened with multiple layers of cheese cloth or a lid with three to five 1-mm holes without cheese cloth, can also be used; however, one must be careful of leakage and crystallisation. Quantity and size of feeders should be adapted to the number of workers requiring food and to the interval between food replenishment. At least 2 devices should be used to reduce the risk of workers starving if one feeder becomes defective, especially if it leaks. Leaky feeders can result in workers starving or drowning; use of ventilation holes or absorptive material on the bottom of cages can prevent the latter. Workers in isolation cages can be individually fed using a micropipette (section 7.8.2).

Refer to section 7.3.2 for a description of providing solid food to caged workers in the laboratory.

7.2.3. Measuring carbohydrate consumption by caged adult workers in the laboratory

Consumption by caged workers can be measured by determining the change in weight or volume of carbohydrate over a given period of time, although most experiments measure the former (Barker and Lehner, 1974). Regardless of method used, consumption should be adjusted for length of feeding period and number of caged individuals to calculate food consumed per honey bee per 24 hours. An easy approach is to simply record consumption every 24 hours, but when this is not possible, recording within 36 hours will suffice, depending upon the size of the feeder and number of caged workers.

To measure average daily carbohydrate consumption per worker for each cage when feeders are not checked every 24 hours:

1. Fill feeder with food.
2. Record mass of food-filled feeder ($MASS_{INITIAL}$).
3. Provide feeder to caged workers; record date and time (hours and minutes) of insertion ($TIME_{INITIAL}$) and number of living caged workers ($WORKERS_{INITIAL}$).
4. Remove feeder after given interval (see section 7.2.4 for frequency of feeder replenishment).
5. Record date and time of removal ($TIME_{FINAL}$), and number of living caged workers ($WORKERS_{FINAL}$).
6. Record mass of feeder ($MASS_{FINAL}$)
7. Determine mass of food consumed (CONSUMED) by subtracting $MASS_{FINAL}$ from $MASS_{INITIAL}$
8. Calculate number of hours (HOURS) the feeder was provided to caged workers using $TIME_{INITIAL}$ and $TIME_{FINAL.}$
9. Calculate hourly cage consumption ($CONSUMED_{HOURLYCAGE}$) by dividing CONSUMED by HOURS.
10. Calculate hourly worker consumption ($CONSUMED_{HOURLYWORKER}$) by dividing $CONSUMED_{HOURLYCAGE}$ by $WORKERS_{FINAL}$; note that consumption is measured for the final living workers, rather than the initial number of living workers or an average of the number of initial and final living workers.
11. Calculate daily worker consumption ($CONSUMED_{DAILYWORKER}$) by multiplying $CONSUMED_{HOURLYWORKER}$ by 24.

Consult section 7.7 to correct for mass of food stuff lost through evaporation.

7.2.4. Replenishing carbohydrates provided to caged adult workers in the laboratory

Care must be taken when renewing carbohydrates because workers are at a higher risk of escaping or being damaged during this time. In theory, 1 ml of 50% (weight/volume) sucrose solution should be adequate for approximately 100 individuals during a 24-hour period because adult workers require 4 mg useable sugar per day to survive (Barker and Lehner, 1974). As worker consumption may vary according

to treatment, at least 5 ml of 50% (weight/volume) sucrose solution for 100 workers should be provided daily to ensure that they do not run out of food.

Carbohydrates should be replenished frequently to ensure they are provided *ad libitum* (i.e. caged workers are never without carbohydrates), or at least every three days to prevent microbial growth or drying when sucrose pastes are provided.

If carbohydrates cannot be provided *ad libitum* to honey bees in isolation cages, individuals can be fed to satiation immediately upon caging, and 16 µl (four 4 µl droplets) of approximately 30% (weight/volume) sucrose solution every 24 hours; this should maintain them for at least one week (Felsenberg *et al.*, 2011).

7.2.5. Recommendations for providing carbohydrates to caged adult workers in the laboratory

The use of a self made sucrose solution is easy, reduces chances of contamination, and depending on type of sugar used, can sustain honey bees for several weeks. Therefore, a good option for providing workers maintained in the laboratory with carbohydrates is to feed 50% (weight/volume) sucrose-tap water solution *ad libitum* (Barker and Lehner, 1978) using a feeder that meets the minimum criteria described previously (section 7.2.2.). Refer to section 7.5 for providing water to caged honey bees.

7.3. Proteins

7.3.1. Types of proteins to provide to caged adult workers in the laboratory

Similar to carbohydrates, source and type of protein (i.e. protein content and amino acid composition) can significantly influence honey bee development, longevity, and immunity (e.g. Haydak, 1970; Pernal and Currie, 2000; Brodschneider and Crailsheim, 2010; DeGrandi-Hoffman *et al.*, 2010; Alaux *et al.*, 2011a). Proteins can be fed to laboratory workers in a variety of forms, although nutritive value, palatability, and digestibility will vary. For example, individuals survived longer (Beutler and Opfinger, 1948) and had higher protein titre levels (Cremonez *et al.,* 1998) when fed pollen collected from the comb (i.e. bee bread) versus pollen traps (i.e. corbicular pollen). Additionally, Peng *et al.* (2012) found that head weight (a surrogate for hypopharyngeal gland size) was larger in young workers fed pollen substitutes compared to various pollen diets.

7.3.1.1. Providing bee bread to caged adult workers in the laboratory

Bee bread, a mixture of fermented pollen, regurgitated nectar, honey, and glandular secretions (Herbert and Shimanuki, 1978), is the natural and most nutritious protein source for young workers. However, it can contain pathogens (Gilliam, 1979) and chemical residues (Genersch *et al.*, 2010; Mullin *et al.*, 2010), and harvesting it is difficult and takes considerable time. A small, metal micro-spatula with a concave blade that is 3-4 mm wide can be used to collect multi-floral bee bread (see section 4.1.8 and the *BEEBOOK* pollination paper by Delaplane *et al.* (2013) for details on identifying multi-floral bee bread). Alternatively, an entire area of cells containing bee bread can be removed from the frame by cutting cross-sections of all cells near their bases. This allows bee bread to be 'popped' out of each cell. Refer to Human *et al.* (2013) in the miscellaneous techniques paper of the *BEEBOOK* for specific instructions on collecting bee bread from colonies. Bee bread can be provided to workers as a 50% (weight/weight) homogeneous paste mixture with sucrose paste (e.g. Cremonez *et al.*, 1998). Refer to section 7.2.1.3 for creating sucrose paste. Quantities may vary, depending upon the nature of the bee bread.

7.3.1.2. Providing corbicular pollen to caged adult workers in the laboratory

Corbicular pollen pellets are units of worker-collected pollen that can be harvested before they are stored in a colony. They provide a common and simple way to provide workers with proteins, and can be collected by outfitting colonies with pollen traps, such as those attached to the hive entrance or those placed under the brood box but above the original colony entrance, as described by Human *et al.* (2013) in the *BEEBOOK* paper on miscellaneous methods. Similar to honey and bee bread, however, corbicular pollen can contain chemical residues and pathogens (e.g. Higes *et al.,* 2008; Mullin *et al.*, 2010), and typically provides relatively fewer proteins than bee bread, possibly because of its reduced digestibility or degradation during storage (*e.g.* Hagedorn and Moeller, 1968; Herbert and Shimanuki, 1978; Dietz and Stevenson, 1980; Cremonez *et al.*, 1998).

To make a 100 g paste containing 90% (weight/weight) fresh corbicular pollen with water (Alaux *et al.* 2010), for example:

1. Add 90 g fresh corbicular pollen to suitable sized glass beaker.
2. Add 10 g tap water to the beaker.
3. Knead using gloved fingers or a spatula until a thick paste is created. Consistency should be similar to soft dough, and it should not ooze.
4. Feed to caged workers, or wrap it in aluminium foil within an air-tight container and store for a few days at -20°C until it is needed.

To make a 100 g paste containing 50% (weight/weight) fresh corbicular pollen with 95% (weight/weight) sucrose candy, for example:

1. Create 50 g of 95% (weight/weight) sucrose candy as described in section 7.2.1.3. in a suitably sized glass beaker.
2. Add 50 g fresh corbicular pollen to the beaker.
3. Knead using gloved fingers or a spatula until a thick paste is created. Consistency should be similar to soft dough, and it should not ooze.
4. Feed to caged workers, or wrap it in aluminium foil and store for a few days at -20°C until it is needed.

7.3.1.2.1. Collecting and storing corbicular pollen to feed to caged adult workers in the laboratory

Based on storage methods described by Pernal and Currie (2000) that successfully maintained honey bee-collected pollen pellets for up to one year without decreasing its nutritional value, the following procedure allows for proper collection and storage of fresh pollen for at least a single field season.

1. Identify a suitable colony to collect pollen from. Refer to section 4.1 for a brief discussion on choosing source colonies for worker collection because pollen should also be collected from healthy colonies.
2. Install a thoroughly cleaned trap (see section 5.2.1. for cleaning equipment using acetone) to collect pollen from incoming foragers sporadically over the course of a few weeks, rather than continuously for more than two days at a time, to ensure colony pollen supplies remains sufficient (see the *BEEBOOK* paper on miscellaneous methods by Human *et al.* (2013)).
3. Carefully separate pollen from other trap debris (i.e. plant material, honey bee body parts) using sterile forceps or a small fine-tipped paint brushe.
4. Separate a subsample of each pollen species based on colour (e.g. Moore and Webb, 1983), and store at –18°C or colder to allow for possible future identification of plant species if needed (see the *BEEBOOK* paper on pollination methods by Delaplane *et al.* (2013)).
5. Homogenise collected pollen to ensure uniform distribution of colony-specific pollen, and store it fresh in air-tight containers at –18°C or colder. Minimize or evacuate air in storage containers.
6. Remove from cold storage only when needed and prepare for feeding as discussed in section 7.3.1.2.

7.3.1.3. Providing pollen substitutes to caged adult workers in the laboratory

Pollen substitutes are artificial diets that do not contain pollen, but rather protein from, for example, soybean, brewer's yeast, milk, or algae (Brodschneider and Crailsheim, 2010). Much like sucrose solution as an artificial source of carbohydrates, these substitutes should contain no honey bee-related pathogens, few chemical residues, and can be more easily standardised among laboratories, especially when purchased from a commercial manufacturer that has strict quality assurance practices.

Both self-made, such as soybean and corn meal patties (e.g. van der Steen, 2007; Ellis and Hayes, 2009), as well as commercially produced substitutes containing essential amino acids, such as Bee-Pro® and Ultra Bee® (Mann Lake Ltd.; Hackensack, USA), Feed-Bee® (Bee Processing Enterprises Ltd; Scarborough, Canada), and MegaBee® (S.A.F.E. R&D; USA) can provide proteins, and possibly other nutrients and vitamins, required by honey bees (e.g. Cremonez *et al.*, 1998; De Jong *et al.*, 2009; Brodschneider and Crailsheim, 2010). Care must be taken because, for example, even soybean flour formulations can vary widely, and ingredients may not be ubiquitously available (Cremonez *et al.*, 1998). Although various homemade recipes exist, the following soy-based pollen substitute was readily consumed by colony honey bees and promoted individual longevity (van der Steen, 2007) (Table 1); however, nutrition tests on caged workers are required.

Table 1. Pollen substitute composition from van der Steen (2007).

Component	**Proportion of total mass**
Soya flower (degreased)	0.143
Beer yeast flour	0.095
Calcium caseinate flour (milk protein 90%)	0.152
Whey protein flour (milk protein 80%)	0.038
Sucrose solution (50% (weight/volume) in tap water)	0.476
Linseed oil	0.095

7.3.2. Feeding devices for providing proteins to caged adult workers in the laboratory

Similar to sugar solution feeding devices, multiple methods exist for providing protein to workers, and the minimum criteria for protein feeding devices are similar to those required for carbohydrate feeding devices. Disposable plastic trays provide the easiest route for providing protein, and can be created by cutting plastic tubes in half to resemble a trough used for feeding livestock that can simply be inserted into cages from the exterior (Fig. 21). Alternatively, a feeder can be created by removing the lower 8 mm tip of a 1.5 ml microcentrifuge tube to reveal a 6-7 mm diameter hole (Fig. 22). This allows workers to enter the feeder and eat the protein upwards. Care must be taken that the protein paste does not leak out the bottom when exposed to incubator conditions (section 6).

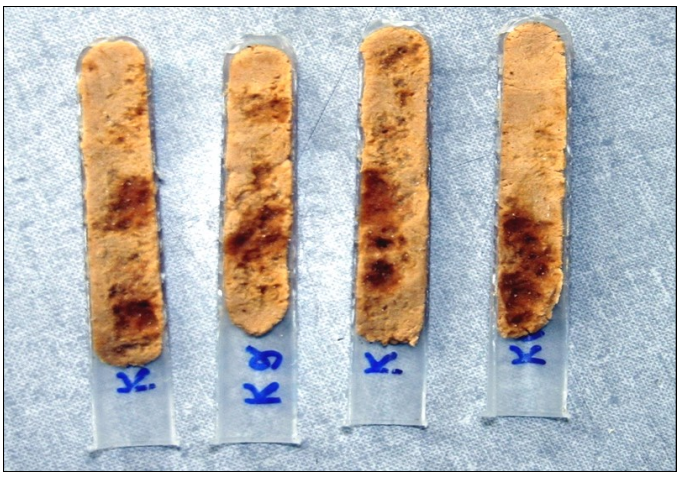

Fig. 21. Protein paste provided to honey bees in 10-ml plastic test tubes cut in half longitudinally. The dark orange-brown areas were moistened by workers during 24 hours in a hoarding cage.

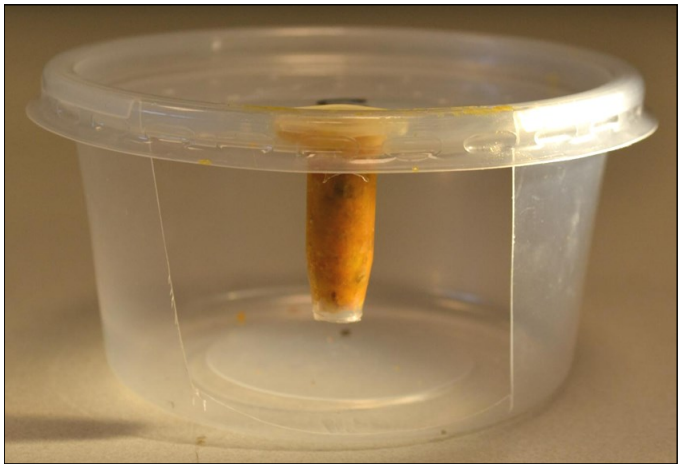

Fig. 22. Protein paste provided in a 1.5 ml micro-centrifuge tube with its base removed to reveal a 6-7 mm diameter hole that allows workers to enter the feeder to consume protein.

7.3.3. Measuring protein consumption by caged adult workers in the laboratory

Consumption can be measured by weighing remaining food, and similar to carbohydrates, should be adjusted to calculate amount consumed per honey bee per 24 hours as detailed in section 7.2.3. It may also be appropriate to calculate quantity of protein consumed, rather than total mass of food stuff providing the protein. This can be determined when the proportion of protein in the food stuff is known. Consult section 7.7 to correct for mass of food stuff lost through evaporation.

7.3.4. Replenishing proteins provided to caged adult workers in the laboratory

Similar to carbohydrates, care must be taken when replenishing proteins to avoid harming caged workers. Feeding pre-trials should be performed to determine quantity needed to ensure workers are fed *ad libitum*. Daily worker consumption should not exceed 3 mg protein; therefore, 3 g of protein paste, at least made from corbicular pollen pellets, should be sufficient to meet daily needs of 100 caged workers. Protein should be replaced at least every three days to prevent drying and microbial growth.

7.3.5. Recommendations for providing proteins to caged adult workers in the laboratory

Under natural conditions, adult workers meet the majority of their protein needs by consuming bee bread within 10 days of emergence (Crailsheim *et al.,* 1992). This protein is vital for proper gland and tissue development, such as the hypopharyngeal and wax glands, flight muscles, and fat bodies (Maurizio, 1959), and consuming it can extend worker longevity beyond that of individuals which only receive carbohydrates (Schmidt *et al*., 1987). Although caged workers can survive extended intervals on carbohydrates alone, providing proteins is recommended when newly emerged or intra-hive workers of an undefined age are caged (see sections 4.2 and 4.3.4 for instructions on how to collect newly emerged and intra-hive workers for laboratory experiments). Protein is not required when flying workers are collected and maintained in the laboratory because they are likely greater than 10 days old and have therefore met their protein consumption demands (Winston, 1987).

Currently we cannot recommend one specific source of protein to provide to caged workers due to lack of data. Multi-floral beebread and corbicular pollen as described previously (sections 7.3.1.1 and 7.3.1.2, respectively) is sufficient for providing proteins as long as it contains minimal pathogens or environmental contaminants. This can be accomplished by sterilising bee products (section 7.6) and collecting from multiple colonies located in non-intensive agricultural areas or from those certified as organic. These multiple colonies ensure that the same, florally diverse pollen is provided to all workers during an entire experiment. Section 4.1 discusses how to select appropriate colonies to collect workers from; similar insights can be used towards the collection of pollen. Alternatively, inexpensive and nutritious pollen substitutes (section 7.3.1.2) that are subject to rigid quality control are ubiquitously available, and may provide a more standardised, sterile protein source to caged workers. Future studies should explore their use, especially those that are fermented by micro-organisms like bee bread to aid their preservation (Ellis and Hayes, 2009).

When used, protein can be provided *ad libitum* using feeders as discussed previously (section 7.3.2), and replaced at least every three days (section 7.3.4). Quality of protein (e.g. nutrition, contamination) should always be considered (see section 7.6 for food sterilisation).

7.4. Lipids, minerals, and vitamins

The importance of lipids, minerals, and vitamins for brood-rearing in a colony is well-known, whereas, in adults it is not (Haydak, 1970; Brodschneider and Crailsheim, 2010). It is likely that reserves stored in the body during development may be used during adulthood (Maurizio, 1959; Haydak, 1970). Honey bees typically receive these nutrients when consuming bee bread (Brodschneider and Crailsheim, 2010), although many protein substitutes can also contain lipids, minerals, and vitamins. Additionally, soluble vitamins of known concentrations can be added to sugar solution, and protein patties or other formulations can be supplemented with lipids, vitamins, and minerals (Herbert *et al*., 1980; 1985; Herbert and Shimanuki, 1978). Little information is available on this subject regarding caged honey bees. More research is needed to better understand effects of lipids, minerals, and vitamins on caged workers, and to determine if they should be provided to individuals as a standard to promote honey bee health in the laboratory. Currently, we recommend to not provide lipid, mineral, and vitamin supplements to caged individuals.

7.5. Water

Water is needed for metabolism and cooling, and is generally obtained by caged workers during ingestion of sugar solutions. In nature, water can also act as an important source of minerals (Brodschneider and Crailsheim, 2010), which can be highly variable depending upon source (WHO, 2005). In North America, for example, tap water provides important sources of calcium, magnesium, and sodium, at least for humans (Azoulay *et al.*, 2001). It is not known how these differences may affect caged workers. Water is essential for maintaining worker honey bees in the laboratory. Carbohydrate solutions containing ≥ 50% (weight/volume) water are sufficient for hydration; if any less is provided, or if only sucrose paste is given, then a separate feeder containing tap water must be offered. Pre-trials for testing feeder leakage may be necessary due to the lower viscosity of water than sucrose solution. Tap water can be boiled to kill harmful micro-organisms, but it should be allowed to return to room temperature before it is given to caged workers.

7.6. Food sterilisation and detoxification

7.6.1. Pathogens and environmental contaminants found in bee products

All bee products, including honey, corbicular pollen, and bee bread, can contain pathogens, environmental contaminants, and agro-chemical residues (e.g. Bromenshenk *et al.*, 1985; Higes *et al.,* 2008; Chauzat *et al.*, 2009; Mullin *et al.,* 2010). A number of methods are available for sanitation of bee products.

7.6.2. Sterilising bee products to destroy pathogens

Bee products can be sterilised to kill pathogens using radiation and temperature treatments.

7.6.2.1. Sterilising bee products to destroy pathogens using radiation

Radiation generally does not alter physiochemical properties of nutrients (Yook *et al.*, 1998) when the appropriate dosage (i.e. treatment intensity and length) is provided (Undeen and Vander Meer, 1990). Greater than 2 kGy of gamma radiation from cobalt^{60} destroyed *N. apis* spores (Katznelson and Robb, 1962), 500 Gy gamma radiation from a caesium^{137} irradiator damaged developmental stages of *N. apis* (Liu *et al.*, 1990), and 10 kGy of high velocity electron-beam radiation effectively sterilised spores of the bacteria *Paenibacillus larvae* and the fungus *Ascophaera apis*, responsible for American foulbrood and chalkbrood disease, respectively (Melathopoulos *et al.*, 2004). Although 3.8 J/cm^2 of 254 nm ultraviolet radiation can reduce viability of *Nosema algerae* spores from moths (Undeen and Vander Meer, 1990), it can also degrade nutrients such as fatty acids (Yook *et al.,* 1998) and may not kill all organisms because the entire food stuff was not penetrated. For pathogen control, the United States Department of Agriculture currently permits a number of fresh or frozen foods destined for human consumption to be irradiated up to a maximum of 5.5 kGy; dried food may be irradiated up to 30 kGy (USDA, 2008).

7.6.2.2. Sterilising bee products to destroy pathogens using temperature

Temperature treatment can be used to sterilise food stuffs; however, nutrient degradation may occur (Barajas *et al.*, 2012). For example, heat treating *N. apis* spores at 49°C for 24 hours will result in their destruction; whereas, freezing *N. ceranae* at -18°C for one week significantly reduces numbers of infective spores (Fries, 2010). Heating honey greater than 49°C should be performed with caution due to the possible production of dangerous levels of toxic hydroxymethylfurfural (HMF) (Brodschneider and Crailsheim, 2010).

7.6.3. Detoxifying bee products to destroy chemicals

Chemicals can be degraded by various methods, such as radiation and temperature treatments; however, rates of degradation vary tremendously depending on compound chemistry, and break down products produced during degradation can also be dangerous to honey bees. Currently, little is known about degradation of chemicals relevant to honey bee health, particularly those in food stuffs.

7.6.4. Recommendations for sterilising and detoxifying bee products fed to caged adult workers in the laboratory

Development of specific protocols to sterilise and detoxify food made from bee products against a broad range of pathogens and environmental contaminants is urgently required. Until then, use of non-honey bee products (sections 7.2.1.2, 7.2.1.3, 7.3.1.3) provide a relatively effective, safe, and standardised approach to supplying food to honey bees. If bee products are fed to caged workers, those products collected from colonies in non-intensive agricultural areas, or from colonies certified as organic, provides a good alternative because they will contain limited chemicals residues and can be sterilised using radiation to kill pathogens.

7.7. Controlling for water evaporation from food provided to caged adult workers in the laboratory

Food consumption is determined by calculating the difference between food provided and food remaining (sections 7.2.3 and 7.3.3). In most cases, evaporation does not need to be considered because all experimental variables should be conserved among treatment groups except for the variable of interest, thereby creating systematic conservative errors among cages.

If water loss from both carbohydrate or protein diets needs to be measured during the course of a study, it can be calculated:

1. Prepare three 'mock' cages ($MOCK_{CAGE}$) cages in the same incubator used to hold experimental cages for food of interest (i.e.

carbohydrates or protein). All conditions for these evaporative control cages should be identical to experimental cages (e.g. type of food provided, frequency of food replacement, type of cage used, incubator maintained in, etc.).

2. Within each $MOCK_{CAGE}$, one 'mock' feeder ($MOCK_{FEEDER}$) should be protected by a breathable mesh; whereas, a second feeder is only provided to allow workers to feed.
3. For each $MOCK_{CAGE}$, calculate average daily mass reduction of $MOCK_{FEEDER}$ feeding device per worker ($MOCK_{BEE}$) according to methods described in sections 7.2.3 and 7.3.3 for measuring carbohydrate consumption.
4. Determine average daily mass reduction per worker among all three cages ($MOCK_{TOTAL}$) using the three $MOCK_{BEE}$ values.
5. Determine loss via evaporation by subtracting $MOCK_{TOTAL}$ from daily per worker food consumed per cage (DAILYWORKER) as determined according to sections 7.2.3 and 7.3.3 for all experi mental cages of interest; negligible negative adjusted con sumptions should be set to zero.

Interestingly, licking or moistening of protein patties by honey bees can adulterate consumption (Fig. 21). At this time, we do not know how to include this behaviour into calculations of food consumption, although it likely has little influence.

7.8. Feeding tests using caged adult workers in the laboratory

Some investigations (e.g. nutrition, toxicology, virology and nosema studies) require that workers receive experimental treatments orally. Typically, the test substance is mixed with food, such as 50% (weight/volume) sucrose solution (section 7.2.1.2). For workers, typical quantities of sucrose solution consumed in nature in a short interval is 50 µl (Seeley, 1994), whereas the honey stomach of drones usually can contain approximately 30 µl (Hoffmann, 1966).

7.8.1. Starving caged adult workers in the laboratory prior to performing a feeding test

Workers are usually starved to ensure that the entire oral treatment is consumed within a short time. So far, no commonly accepted method for starving individuals prior to oral application of a treatment exists; however, within fields of study there are some consistencies. For example, most experimental laboratory investigations of *Nosema* starve groups of young workers for two to four hours (e.g. Fries *et al.*, 1992; Malone and Stefanovic, 1999; Higes *et al.*, 2007; Maistrello *et al.*, 2008); starvation for this length is also recommended by Fries *et al.* (2013) in the *BEEBOOK* paper describing methods used to study nosema in honey bees. Similarly, up to two hours of starvation is recommended for acute, oral toxicity experiments (OECD, 1998). Amount of food in the honey stomach will no doubt influence length of required starvation time, and resilience to starvation will likely depend on type of collected workers (e.g. age, health, etc.), and if they were starved individually or in a group.

Future studies should investigate effects of both short and long-term starvation on honey bees, in addition to the influence of honey bee condition, age, and subspecies. Generally, groups of adult workers should be starved for no more than four hours to ensure rapid consumption of a test substance. Workers starved in isolation should be without food for less time – no longer than two hours. Individuals starved for any longer are more likely to be injured or to die. Starved honey bees that do become impaired (e.g. behaviourally) or that exhibit other unusual signs should be discarded from experimental studies. Pre-trials will determine the minimum length of starvation period needed to consistently induce feeding of entire food treatment quickly.

7.8.2. Feeding a liquid test substance to individual adult workers in the laboratory

Individual feeding is used when specific, known quantities of test substance are required to be ingested by individual workers. Although precise, individual feeding can be extremely time-consuming and may inadvertently limit sample size.

The easiest way to orally feed workers liquid test substances individually is to provide a micropipette filled with a known quantity of test substance to an individual as detailed below. A specific quantity (i.e. the same volume for each experimental worker) between 3-10 µl should be provided. This will ensure that all workers can easily consume the same volume of homogeneously mixed test substance.

1. Because some workers may not feed, it is appropriate to starve more individuals than will be required for the experiments. Pre-trials testing starvation times and test substance consumption will help determine how many workers will be needed.
2. Remove a starved individual worker from its cage using a forceps by gently grasping a leg. Refer to Human *et al.*, (2013) in the *BEEBOOK* paper discussing miscellaneous methods for details on how to handle adult honey bees.
3. Gently grasp the wings together at their base using the thumb and index finger so that her mouthparts are exposed (i.e. wings facing down) and her stinger is pointing away from your body (Fig. 23).
4. Vortex the food test substance for 5 seconds.
5. Feed a specific volume (i.e. a volume between 3-10 µl) of liquid test substance to the worker using a micropipette, which allows for a precise volume to be administered. Place the end of the loaded pipette tip in front of the individual's mouthparts or beneath the mandibles in front of the maxillae and create a small droplet at the open end of the pipette tip to promote feeding (Fig. 23). Additionally, the pipette tip can be gently placed against an antenna when the honey bee is reluctant to feed.

Fig. 23. A worker honey bee being individually fed using a micropipette. Note that the individual is held by gently squeezing its wings between the index finger and thumb, and that the distal part of the abdomen is pointed in such a way that the honey bee cannot sting the handler.

6. Provide the remaining test substance by depressing the pipette plunger gently to ensure that nothing spills when the individual begins to feed. Discard the individual and start over using a new worker if she does not consume all of the test substance within one minute.
7. Place the fed worker in an appropriate cage (section 5) with food (section 7) maintained under proper incubator conditions (section 6).
8. If needed, isolate the worker for 20-30 minutes to observe regurgitation or to ensure that none of the test substance is passed to another worker via trophallaxis. Isolation is not required when individually feeding queens and drones because they are not food providers (Crailsheim, 1998), and therefore will not discard the test substance to another individual. Orally transmitted pathogens take fewer than 15 minutes to enter the ventriculus after ingestion (Kellner and Jacobs, 1978; Verbeke *et al.,* 1984).

Although typically less efficient, individuals withheld in isolation cages, such those shown in Figs 17 and 18, can also be fed a test substance using a micropipette, and may minimize the handling of honey bees.

7.8.3. Feeding a liquid test substance to groups of caged adult workers in the laboratory

In contrast to feeding a liquid test substance to individuals, group-feeding has fewer logistic and time constraints. It mimics consumption and transfer of food among honey bees in a colony via trophallaxis because food is typically consumed by only a small proportion of workers but ultimately shared among nearly all worker nest-mates within 24 hours (Nixon and Ribbands, 1952; Crailsheim, 1998).

Although not well studied, the primary disadvantage of group feeding a test substance is its potential unequal distribution among individuals over time (Furgala and Maunder, 1961). Although many factors may influence food consumption, such as parasitism (Mayack and Naug, 2009), quantity of honey bees and level of starvation will most importantly dictate volume of test substance to provide. Generally, ten workers can consume 100 - 200 µl of 50% sucrose solution in 3-4 hours (OECD, 1998), or at an individual rate of 2.5 - 6.6 µl per hour. To group feed workers a known quantity of inoculum, a single feeding device containing a minimal amount of test substance should be used to ensure all contents are consumed in a timely fashion. Generally, the entire volume should be consumed in less than 24 hours, but exact duration of consumption should be determined by the specific experiment. The test substance should be replaced with standard food when the total volume of the test substance is ingested; constant vigilance is required to ensure that workers do not go without food. One should assume equal consumption by all caged workers when determining ingestion of test substances. For example, 1,000,000 *N. ceranae* spores are required to inoculate 30 workers with 33,333 spores each.

7.8.4. Feeding a solid test substance to groups of adult workers caged in the laboratory

Test substances can also be provided orally to a group of caged workers in a solid form, such as in 95% (weight/weight) sucrose paste (section 7.2.1.3). See section 7.8.3. for a discussion on feeding a group of workers in a single cage. Ensure the solid test substance is well mixed and homogeneous, and perform a pre-trial to determine how much sucrose paste is required.

7.8.5. Recommendations and considerations for oral exposure of a test substance to caged adult workers in the laboratory

Choice of whether to inoculate workers individually or as a group will mainly depend upon the particular experiment. Few investigations have compared results from individual versus group feeding of a test substance, although it is clear that both can be effective. For example, Tanner *et al.* (2012a) demonstrated no difference in individual versus group feeding of intra-host *N. ceranae* spore development 14 days post-inoculation. Research should also examine homogeneity of test substances, especially suspensions containing particles such as *Nosema* spores that may settle in liquid. In these cases, a test substance fed as a solid may ensure a more even distribution of particles.

8. Queens and drones

So far we have discussed how to properly maintain worker honey bees under *in vitro* laboratory conditions, mainly ignoring queens and drones. Because workers are generally required to provide food via trophallaxis to both of these groups, many of the methods described

above, such as incubator conditions, cage designs, and nutrition, are also valid for maintaining adult queens and drones in the laboratory. When choosing worker attendants, researchers must also consider that workers can horizontally transmit pathogens to both drones and queens (e.g. Higes *et al.*, 2009).

8.1. Maintaining queens under *in vitro* laboratory conditions

Adult queens can be maintained safely in the laboratory when kept in cages with workers collected from brood frames from the same source colony as the queen. For up to five days, a queen can be placed in a standard queen cage provided with sucrose candy *ad libitum* and four to seven workers (Fig. 24); however, for longer intervals a queen should be maintained with at least 10 workers in a standard worker hoarding cage as discussed in section 5. To obtain and maintain virgin queens in the laboratory, a cell from which a queen is expected to emerge from within two to four days can be placed in a hoarding cage with workers (Alaux *et al.*, 2011b) under appropriate incubation conditions described in section 6. When performing experiments, it is important for researchers to consider nutrients that should be provided to caged queens and workers because of the importance of protein to tissue and organ development (*e.g.* Hersch *et al.*, 1978; Pernal and Currie, 2000). More detailed instructions on rearing and maintaining queens can be found in the *BEEBOOK* paper on queen rearing and selection (Büchler *et al.*, 2013).

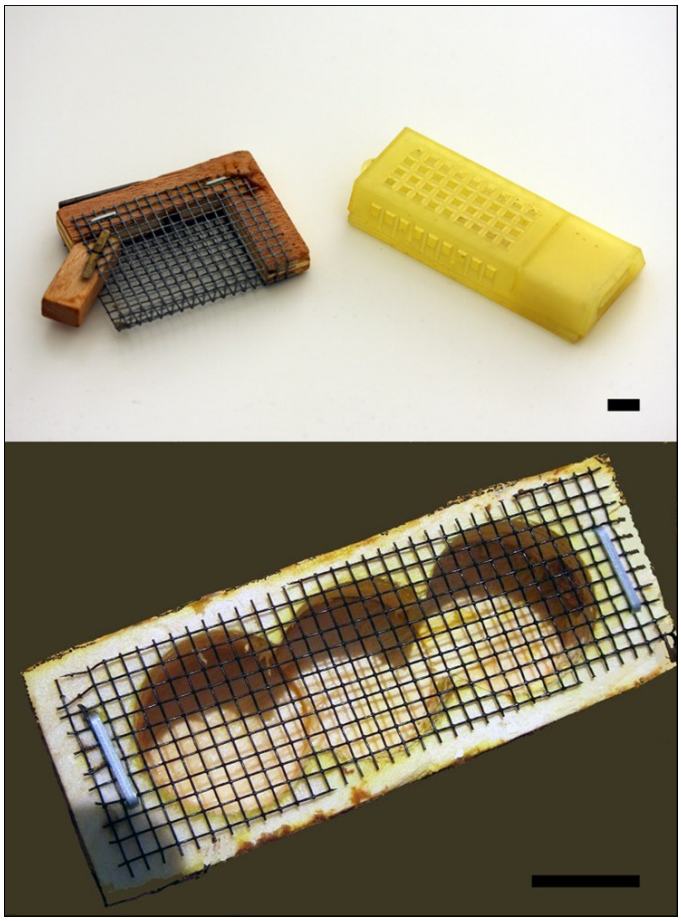

Fig. 24. Standard queen cages suitable for maintaining a queen and approximately five nurse worker honey bees safely in a growth chamber for up to five days when sucrose candy is provided *ad libitum*. Top and bottom images not equal in scale; black lines denote ~0.9 cm.

8.2. Maintaining drones under *in vitro* laboratory conditions

Similar to queens, drones should be maintained in the laboratory with workers collected off brood frames. Preferably, these workers should come from the same colony as the drones to facilitate the latter's acceptance. Additionally, attention must be paid to the type of nutrients provided to caged drones and workers because of the potential importance of protein to development of tissues, including gonads (Jaycox, 1961). Unlike queens, multiple drones can be kept in the same cage, and at a 2:1 drone:worker ratio (Jaycox, 1961; Huang *et al.,* 2012). This will ensure that drones survive at least until they reach maturity, approximately 8-9 days post-emergence (Jaycox, 1961). If caged individuals die during the experiment, one should consider maintaining this drone:worker ratio by adding or removing workers.

Because of the affinity of the parasitic mite *Varroa destructor* to drones, researchers must also consider the influence of parasitism during development when designing experiments. Drones should be maintained in conditions previously recommended for adult workers because they exhibit a similar thermo-preference (Kovac *et al.*, 2009). However, future studies should evaluate alternative temperature and feeding regimes when evaluating drone reproductive traits because of the sensitivity of sperm production. For example, Jaycox (1961) recommended that drones be kept between 31 and 34°C, and suggested that drones can be caged without workers when appropriate feeding devices provide honey rather than sucrose because of drones' difficulty to invert sugars. General methods for maintaining drones more appropriately in the laboratory urgently need development because of their greater sensitivity to *in vitro* conditions (Tanner *et al.*, 2012b).

9. Conclusions and future directions

In this paper we have primarily discussed methods for maintaining adult worker honey bees *in vitro* in the laboratory. The main purpose for providing these recommendations is to promote standardisation of research methods that will facilitate comparison of data generated by different laboratories. Although methods for maintaining adult workers *in vitro* are typically capable of sustaining workers for many weeks, the real issue lies in creating an experimental environment that can produce biologically relevant data. Honey bees are highly social organisms; no doubt placing 30 workers in a cage without a queen will have consequences for their behaviour and physiology. Additionally,

proper nutrition is paramount to immune responses of all animals, including honey bees (Alaux *et al.*, 2010), yet the majority of laboratory experiments feed carbohydrates only, ignoring key nutrients honey bees normally consume in the natural environment (Brodschneider and Crailsheim, 2010). Researchers should also assume that laboratory settings provide a relatively stressed environment compared to the colony arena, which has many buffering mechanisms to fend off external threats. Therefore, most studies performed in the laboratory should represent the first step in performing hypothesis-driven research, with further studies carried out under natural "field" conditions.

Ultimately, a laboratory setting can provide an environment in which one can perform controlled investigations using honey bees to test falsifiable hypotheses using appropriate experimental designs. Given the potential influence of the myriad variants in a laboratory, researchers must maintain honey bees under appropriate and repeatable conditions, and should always provide sufficient details about their experiments so that data can be more easily interpreted and compared.

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