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REVIEW ARTICLE

Standard methods for physiology and biochemistry research in *Apis mellifera*

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Summary

Despite their tremendous economic importance, and apart from certain topics in the field of neurophysiology such as vision, olfaction, learning and memory, honey bees are not a typical model system for studying general questions of insect physiology. The reason is their social lifestyle, which sets them apart from a "typical insect" and, during social evolution, has resulted in the restructuring of certain physiological pathways and biochemical characteristics in this insect. Not surprisingly, the questions that have attracted most attention by researchers working on honey bee physiology and biochemistry in general are core topics specifically related to social organization, such as caste development, reproductive division of labour and polyethism within the worker caste. With certain proteins playing key roles in these processes, such as the major royal jelly proteins (MRJPs), including royalactin and hexamerins in caste development, and vitellogenin in reproductive division of labour and age polyethism, a major section herein will present and discuss basic laboratory protocols for protein analyses established and standardized to address such questions in bees. A second major topic concerns endocrine mechanisms underlying processes of queen and worker development, as well as reproduction and polyethism, especially the roles of juvenile hormone and ecdysteroids. Sensitive techniques for the quantification of juvenile hormone levels circulating in haemolymph, as well as its synthesis by the *corpora allata* are described. Although these require certain instrumentation and a considerable degree of sophistication in the analysis procedures, we considered that presenting these techniques would be of interest to laboratories planning to specialize in such analyses. Since biogenic amines are both neurotransmitters and regulators of endocrine glands, we also present a standard method for the detection and analysis of certain biogenic amines of interest. Further questions that cross borders between individual and social physiology are related to energy metabolism and thermoregulation. Thus a further three sections are dedicated to protocols on carbohydrate quantification in body fluid, body temperature measurement and respirometry.

Métodos estándar para la investigación de la fisiología y bioquímica de *Apis mellifera*

Resumen

A pesar de su enorme importancia económica, y aparte de ciertos temas en el campo de la neurofisiología, tales como la visión, el olfato, el aprendizaje y la memoria, las abejas no son un sistema modelo típico para el estudio de cuestiones generales sobre la fisiología de los insectos. La razón de ello es su forma de vida social, lo que las diferencia de un "insecto típico" y que durante la evolución social, se ha traducido en la reestructuración de ciertas vías fisiológicas y bioquímicas propias de este insecto. Como era de esperar, las preguntas que han atraído mayor atención por parte de los investigadores que trabajan en la fisiología y la bioquímica de la abeja melífera, son en general temas relacionados específicamente con la organización social, tales como el desarrollo de las castas, la división reproductiva del trabajo y el

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polietismo de la casta obrera. Dadas aquellas proteínas que juegan un papel clave en estos procesos, como la proteína principal de la jalea real (MRJPs), incluyendo a la royalactina y las hexamerinas en el desarrollo de las castas, y la vitelogenina en la división reproductiva del trabajo, el polietismo vital, una importante sección de este documento presentará y discutirá protocolos básicos de laboratorio establecidos y estandarizados para el análisis de dichas proteínas para abordar esas cuestiones en las abejas. Un segundo tema importante se refiere a los mecanismos endocrinos subyacentes en los procesos de desarrollo de la reina y de las obreras, así como la reproducción y el polietismo, especialmente el papel de la hormona juvenil y los ecdisteroides. Se describen algunas técnicas para la cuantificación de los niveles de hormona juvenil circulantes en la hemolinfa, así como su síntesis por el *allata corpora*. Aunque éstos requieren cierta instrumentación y un grado considerable de sofisticación en los procedimientos de análisis, se consideró que la presentación de estas técnicas podría ser de interés para los laboratorios que planifiquen especializarse en este tipo de análisis. Dado que las aminas biogénicas son neurotransmisoras y reguladoras de las glándulas endocrinas, también presentamos un método estándar para la detección y el análisis de ciertas aminas biogénicas de interés. Otras preguntas entre la fisiología individual y la social están relacionadas con el metabolismo energético y la termorregulación. Así, una sección final está dedicada a los protocolos para cuantificar hidratos de carbono en el fluido corporal, la medición de la temperatura corporal y la respirometría.

西方蜜蜂生理学和生物化学研究的标准方法

蜜蜂不是研究昆虫生理学一般问题的典型模式， 虽然蜜蜂具有重要的经济价值，并在诸如视觉、味觉、学习及记忆等神经生理学领域有特殊性，原因在于它们的社会性生活方式，在社会性进化过程中蜜蜂重建了某些生理学通路和生物化学特性， 使其有别于“典型的昆虫”。 毫无疑问，蜜蜂生理学和生物化学方面的研究多集中在蜜蜂的社会性组织结构，如：级型发育、劳动分工以及工蜂行为多态性。在这些过程中，某些蛋白质发挥着关键作用，如王浆主蛋白（MRJPs），包括“成王”蛋白（royalactin）和储存蛋白（hexamerins）影响级型发育，卵黄蛋白原影响生殖和日龄相关的劳动分工。所以本文将主要阐述和讨论用于建立和标准化蜜蜂蛋白质分析实验的基本实验指南。其次，还阐述了与工蜂和蜂王发育相关的内分泌调控机制，包括繁殖和行为多态性，特别是保幼激素和蜕皮激素的作用。还介绍了定量淋巴液中保幼激素水平以及咽侧体合成量化的灵敏技术。尽管这需要特殊的仪器设备，并且分析过程相当复杂，但我们认为对计划从事这些分析的实验室来说提供这些技术很有价值。由于生物胺既是神经传导物质又是内分泌腺的调节器，所以我们还提供了一些生物胺的测定和分析的标准方法。由个体水平的生理学研究跨越到群体生理学研究，关键在于探明能量代谢和温度调节的规律。所以本文最后一部分给出了蜜蜂体液中碳水化合物的量化、体温测量和呼吸测量的实验方案。

Keywords: haemolymph, protein, electrophoresis, SDS-PAGE, western blotting, immunofluorescence, sucrose, trehalose, juvenile hormone, radioimmunoassay, gas chromatography, *corpora allata*, temperature, thermosensors, thermography, radiation, humidity, operative temperature, respiration, energetics, gas exchange, respirometry, oxygen consumption, calorimetry , COLOSS, *BEEBOOK*, honey bee

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1. Protein analysis for honey bee samples

1.1 Introduction

Obtaining protein profiles of an organism is the basis for assessing several aspects of biological processes. The protein content and protein composition in haemolymph, whole body or specific tissue extracts can provide valuable information on developmental stage, reproductive potential, aging processes, health status and correlated processes. Furthermore, quantitative analysis of protein content could be the starting point for standardizing or normalizing measures on other physiological, biochemical, or morphological parameters.

The first step in any protein analysis is usually the assessment of total protein content in a given sample, so as to guide further studies, especially comparative ones. As such, accurate measurement of protein concentration is critical for any further calculations such as, representation of specific proteins in a sample and, even more so, when determining enzyme activity. Errors in the calculation of protein concentration will tend to amplify overall errors in any such further estimates.

We selected a series of classical protocols currently used for an accurate measure of protein content of samples with different natures. The simplest method for quantifying protein content is spectrophotometry at 280 nm. However, this approach is not very reliable or sensitive compared to the two principle approaches that are detailed. The first approach is the Bradford Assay, which is based on the differential binding of a staining compound (Coomassie) through ionic interactions between sulfonic acid groups and positive amine groups on proteins (Bradford, 1976). The second is the bicinchoninic acid (BCA) method, which gained importance as a means for quantification of detergent extracted protein samples (Smith *et al.*, 1985). The Coomassie method is cheaper and very well suited for quantifying haemolymph proteins, but it is sensitive to higher detergent concentrations, as typically used for extracting proteins from tissue. In this case the BCA method is preferable. Other frequently used methods, such as that using Biuret-Folin-Ciocalteu reagents (Lowry *et al.*, 1951) are equally sensitive as the Bradford or BCA methods, but are more laborious, and it is only for the latter reason that we do not describe the Lowry method here.

In contrast to the determination of total protein content, the analysis of protein composition can be done by a plethora of methods and their respective variants. For this reason we decided to focus on just a few which can easily be established in any laboratory with basic equipment for analytical biochemistry and using low cost reagents. These are an electrophoretic separation of proteins according to their molecular mass (actually the Stoke radius of denatured proteins), based on the original method by Laemmli (1970), and two immunological methods (Western blot analysis and rocket immunoelectrophoresis) for the detection of specific proteins in complex mixtures. Each of these methods have been frequently utilized in research on honey bees. Western blot analysis has now become a gold-standard method for identifying specific proteins, but when emphasis is on more precise quantification, rocket immunoelectrophoresis is more precise.

Notwithstanding, the methods outlined here are ones that can fairly easily be implemented in any laboratory, as they do not require sophisticated equipment. Obviously, more advanced methods are available, starting from two-dimensional electrophoresis (2DE) to ever more sophisticated and high throughput proteomics analyses. In 2DE, proteins are usually first separated by isoelectric focusing and then by SDS-PAGE in the second dimension. Such gels have much higher resolution than one-dimensional gels, and spots detected in such gels can be retrieved for amino acid sequencing by Matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) analysis followed by comparison of amino acid sequences to proteome databases, e.g. MASCOT. 2DE methods have, for instance, been applied to study the honey bee haemolymph proteome (Chan and Foster, 2008; Boegaerts *et al.*, 2009), and MALDI-TOF proteomics analyses have also been applied to a variety of questions in honey bee biology (Santos *et al.*, 2005; Collins *et al.*, 2006; Li *et al.*, 2008).

1.2. Quantification of total protein content in samples

A crucial aspect when assaying protein concentration is the selection of an assay compatible with the sample. As such, whilst simple and well suited for analysing haemolymph protein content, the main disadvantage of Coomassie-based protein assays is the interference of certain detergents at concentrations routinely used to solubilise membrane proteins.

1.2.1. The Bradford assay

The Bradford assay for protein quantification is a popular protein assay because it is simple, rapid, inexpensive, and sensitive. It is based on the direct binding of Coomassie Brilliant Blue G-250 dye (CBBG) to proteins at arginine, tryptophan, tyrosine, histidine, and phenylalanine residues.

1. Prepare the protein reagent (Bradford reagent) by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol.
2. Add 100 ml of 85% (w/v) phosphoric acid.
3. Dilute to 1l with distilled water
4. Let the solution stir overnight to assure maximal dissolution.
5. Filter (e.g. through Whatman #1 paper) and store in a dark bottle.
6. Prepare a standard curve from a 1 mg/ml of bovine serum albumin (BSA fraction V) stock solution by pipetting 1, 2, 5, 7, 10, 15 and 20 µl of this solution into glass test tubes. It is of importance not to extend the range of the standard curve beyond 20 µg/µl when using BSA to guarantee that measurements are within a linear range.
7. Complete each tube with distilled water to a final volume of 20 µl.
8. Prepare these in triplicates.
9. Prepare blank samples containing 20 µl of distilled water.
10. Also prepare triplicates from 20 µl of each unknown sample, that must be adequately diluted (this must be done empirically) to give measurements within the range of the standard curve.
11. Add 1 ml of Bradford reagent to blanks, standards and samples, vortex, leave for 2 min at room temperature and transfer the solution to disposable plastic cuvettes.
12. Set spectrophotometer to a wavelength of 595 nm.
13. Absorbance should be measured after 2 min and before 1h from the moment that Bradford reagent was added.
14. Average the absorbance readings of each of the triplicates and subtract blanks from standards and samples.
15. Plot absorbance values of the standard curve samples against their protein concentration (µg/µl).
16. Determine the concentration of the unknown samples by linear regression.

1.2.2. The bicinchoninic acid (BCA) assay

The BCA assay (Smith *et al.*, 1985) uses bicinchoninic acid (BCA) in a reaction forming Cu^{+} from Cu^{2+} by the Biuret complex in alkaline solutions of protein. The advantage of this assay is its high tolerance towards the presence of detergents in protein extracts.

1. Prepare Reagent A (aqueous solution containing 1% of 2,2'-Biquinoline-4,4-dicarboxylic acid disodium salt, 0.16% of sodium tartrate, 0.4% sodium hydroxide, 2 % of $Na_2CO_3 \cdot H_2O$, 0.95% $NaHCO_3$) .
2. Adjust pH to 11.25.
3. Prepare Reagent B (4% $CuSO_4 \cdot 5H_2O$ in deionized water). These reagents are stable indefinitely when kept in dark bottles at room temperature.
4. Standard working reagent (S-WR) should be prepared weekly or as needed by mixing 50 volumes of Reagent A with 1 volume of Reagent B.
5. Prepare a convenient standard curve using a solution 1 mg/ml of bovine serum albumin (BSA fraction V) in either isotonic saline or, in the case of any possibly interfering substance (e.g. sodium dodecyl sulphate, SDS), in a solution containing this particular substance.
6. Prepare blanks and triplicates of standards and samples (as described above in step 6 of the Bradford assay).
7. Add 20 volumes of S-WR per volume of sample (e.g. add 950 µl S-WR to a 50 µl sample).
8. Add 1 ml of S-WR to each.
9. Vortex well for a few seconds.
10. Incubate the samples for 30 min at 37ºC.
11. Cool samples to room temperature.
12. Transfer to disposable plastic cuvettes.
13. Set spectrophotometer to a wavelength of 562 nm.
14. Read standard curve and unknown samples.
15. Average the absorbance readings of each of the triplicates and subtract blanks from standards and samples.
16. Plot absorbance values of the standard curve samples against their protein concentration (µg/µl).
17. Determine the concentration of the unknown samples by linear regression.
18. Make sure that the standard curve is linear and that unknown samples are within range.

1.3. One-dimensional SDS gel electrophoresis of proteins

Electrophoresis is used for investigating complex mixtures of proteins by separating these according to their mobility in an electric field. It can be used to analyse subunit composition of certain proteins, to verify homogeneity of protein samples, and to purify proteins for use in further applications.

Polyacrylamide gels are the most commonly used matrices in electrophoretic separations being less costly than agar or agarose gels and providing a very broad range of options for defining matrix pore size (the sieving properties of the gel) through selecting appropriate

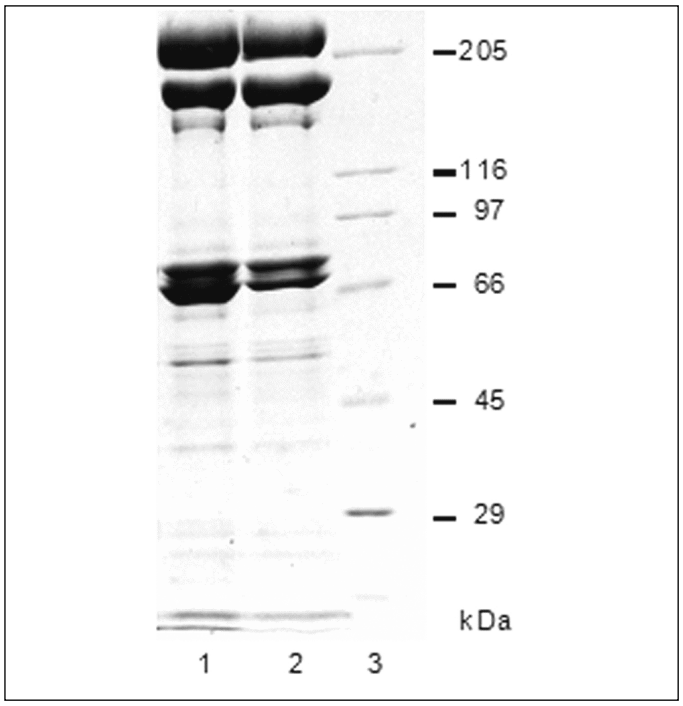

Fig. 1. Haemolymph protein patterns of *Apis mellifera* workers separated by SDS-PAGE (7.5 %), lane 1: 4-day-old worker; lane 2: 6-day-old worker; lane 3: molecular mass marker. Gel stained with Coomassie Brilliant Blue. Modified from Bitondi and Simões (1996). Copyright *Journal of Apicultural Research*.

proportions of polyacrylamide/bisacrylamide and water in the gel mix. In polyacrylamide electrophoresis (PAGE), proteins are separated according to charge and molecular mass/molecule structure. The use of sodium dodecyl sulphate (SDS) as detergent for eliminating differences in charge, the reduction of disulfide bonds by treatment with a reducing reagent, such as ß-mercaptoethanol or dithiothreitol (DTT) and heat to denature protein structure, were innovations by Laemmli (1970). This created the widely used SDS-PAGE protocols for separating proteins according to their Stoke's radius, commonly referred to as molecular mass (not molecular weight, as weight is dependent on gravity).

Unlabelled proteins separated by PAGE are typically detected by staining either with Coomassie Brilliant Blue or with silver salts. Coomassie Brilliant Blue binds nonspecifically to proteins but not the gel, thereby allowing visualization of the proteins as discrete blue bands within a translucent gel matrix. Observe that Coomassie Brilliant Blue G-250 is used in the Bradford assay, but it is Coomassie Brilliant Blue R-250 which is used for staining gels. These are different reagents, so be careful to use the correct one for each application.

Silver staining, although more laborious, is significantly more sensitive, but it may present problems when quantification of protein bands in gel documentation systems is the aim. The coloration of silver-stained bands is not uniform and bands of high protein content may in fact invert colour intensity and appear transparent.
Although there are a plethora of variations of the original SDS-PAGE protocol (Laemmli, 1970) adapting the method to specific problems, we describe a protocol commonly used to assess the haemolymph composition (Fig. 1) of honey bee larvae and adults (Pinto *et al.*, 2000; Barchuk *et al.*, 2002; Guidugli *et al.*, 2005; Bitondi *et al.*, 2006). A major variation in this protocol compared to the original Laemmli protocol is that there is no SDS in the gel, but only in the buffers. This avoids the precipitation of SDS in the gel matrix when running thin gels at low temperatures, conditions used to improve separation. After protein staining with Coomassie Brilliant Blue and scanning the gels on a gel documentation system, it is further possible to quantify specific proteins by Image J software (http://rsbweb.nih.gov/ij/index.html) or by commercial software implemented in gel documentation systems. Gels stained with silver salts are not appropriate for such quantitative analyses as silver staining does not follow linear characteristics.

1.3.1. Preparing samples

Ideally, samples should contain about 1 - 10 µg of total protein to give optimal results, thus protein content of the samples should be assessed by one of the methods described above (Bradford or BCA, section 1.2). Haemolymph proteins are usually best separated in gels with a 7.5% acrylamide concentration.

1. Prepare the sample buffer by dissolving 1.51 g Tris, 20 ml glycerol in 35 ml of double distilled water (ddH_2O).
2. Adjust pH to 6.75 with 1 N HCl.
3. Then add:
   - 3.1. 4 g SDS,
   - 3.2. 10 ml 2-mercaptoethanol,
   - 3.3. 0.002 g bromophenol blue,
   - 3.4. ddH_2O to a final volume of 100 ml.
4. Prepare protein samples (haemolymph) by adding the sample to the sample buffer in a 1:1 (v/v) ratio.
   Very diluted samples may require a 2X concentrated sample buffer to make an adequate volume of 10 – 15 ml.
5. Prepare a sample containing the molecular mass markers, following the manufacturer's instructions.
   Use the same sample buffer (step 1) as that used for your samples.
6. Heat samples and the molecular mass marker sample in a boiling water bath for 1 – 3 min to denature protein structure.
   Perforate tops of Eppendorf tubes to avoid popping of the lid and spilling of the sample as internal pressure increases with heating.
7. Cool on ice for a few minutes.
8. Spin in a refrigerated tabletop centrifuge at maximum speed for 5 min.
9. Use supernatant only for application to the gel, as the precipitate may contain protein and nucleic acid aggregates which may cause streaking along the separation path.

User safety: Mercaptoethanol is an irritant and a foul smelling compound. It can be substituted with dithiotreitol.

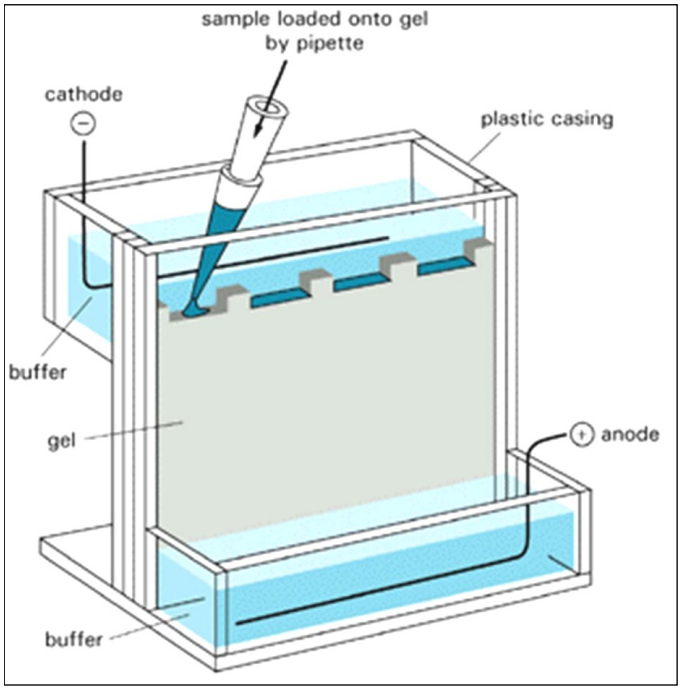

Fig. 2. Setup of a vertical SDS-PAGE system. Observe polarity settings.

1.3.2. Preparing and running vertical slab gels

This protocol is designed for a vertical slab gel with dimensions 100 x 120 x 0.9 mm (Fig. 2). For thicker gels or other gel sizes, the volumes of separating and stacking gels and the electric current must be adjusted accordingly.

1. Prepare 100 ml of an acrylamide stock solution containing
   1.1. 30% (w/v) acrylamide
   1.2. 0.8% (w/v) *N,N´*-methylene bisacrylamide in ddH_2O.

This solution can be stored refrigerated for 2-3 weeks.

2. Prepare a 1.5 M Tris buffer with a pH of 8.8, for this add:
   2.1. 18.15 g of Tris-base to
   2.2. 80 ml of ddH20.
   2.3. Use 1N HCl to adjust pH to 8.8.
   2.4. Complete volume to 100 ml.
3. Prepare a 0.25 M Tris buffer with a pH of 6.8, for this add:
   3.1. 3 g of Tris-base to
   3.2. 80 ml of ddH20
   3.3. Use 1N HCl to adjust pH to 8.8
   3.4. Complete volume to 100 ml.

These buffers for the preparation of the separation and stacking gels, respectively, can be stored refrigerated.

4. Prepare 1 l of electophoresis buffer: dilute in 1 l of distilled water
   4.1. 3.03 g Tris.
   4.2. 14.4 g glycine.
   4.3. 1 g SDS.

A polyacrylamide gel with a 7.5% separating gel is then prepared and run in the following sequence:

5. Mix
   5.1. 2.5 ml of the acrylamide/bis-acrylamide stock solution,
   5.2. 5 ml of the 1.5 M Tris buffer (pH 8.8),
   5.3. 2.3 ml ddH_2O.
6. Stir gently so as not to introduce air bubbles, as oxygenation may impede polymerization.
7. Add 190 µl of 1% ammonium persulphate (APS) solution and 40 µl of *N,N,N′,N′*-Tetramethylethylenediamine (TEMED).

These are the starters for the polymerization process.

8. Quickly mix the reagents and immediately pour the solution into the cassette formed by the two glass plates sandwiched over sealing spacers.

Leave sufficient space for later pouring the stacking gel on top.

9. Carefully overlay the gel with water to guarantee a smooth and straight surface.
10. Wait for about 30 min until the gel is completely polymerized. If it does not polymerize in due time, your APS solution is probably too old.
11. After polymerization is completed, pour off the water and carefully remove any remaining water with filter paper, but avoid touching the gel surface.
12. Prepare a stacking gel (4.26%) by mixing
    12.1. 0.375 ml of the acrylamide/bisacrylamide stock solution.
    12.2. 1.38 ml of the 0.25 M Tris buffer (pH 6.8) .
    12.3. 0.825 ml ddH_2O.
13. Stir gently so as not to introduce air bubbles.
14. Add 51 µl of a 5% ammonium persulphate (APS) solution.
15. Add 10 µl TEMED.
16. Pour the stacking gel on top of the separating gel.
17. Insert a Teflon comb with blunt-ended teeth for creating the sample application wells.

Be careful to avoid introducing air bubbles.

18. Allow the gel to polymerize completely at room temperature. Once polymerized, gels can be stored in the refrigerator for up to a few days, but make sure to wrap the glass plate-gel sandwich with household PVC film.
19. Mount the gel sandwich in your vertical electrophoresis apparatus.
20. Fill the tanks with electrophoresis buffer.
21. Carefully remove the Teflon comb pulling up evenly by a small amount on each side.
22. Using a micropipette with long tips or a Hamilton-type syringe load the samples into the wells.

The sample solution will be more dense than the running buffer, and will displace the latter when pipetted into the comb wells; the added bromophenol blue allows this to be visualized.

23. Connect the electrophoresis apparatus to a power supply, make sure that polarity is correct (see Fig. 2).
24. Carry out the electrophoresis run at a constant current of 15 mA, preferably in a cold room or refrigerator, until the bromophenol blue front reaches the desired position, usually 0.5 cm above the gel bottom.

25. Switch off power supply and remove cables.
26. Dismount the cassette, remove the stacking gel and carefully slide the gel off the glass plate into the dish containing the fixation/staining solution.

General advice: Ammonium persulphate (APS) decomposes within a short time, so a fresh working solution should be prepared weekly. Both APS and TEMED are starters for the polymerization process, so make sure to pour gels quickly after these compounds are added.

User safety: Acrylamide and bisacrylamide are neurotoxic compounds, so use protective equipment (fume hood, safety glasses and gloves) when preparing and handling any acrylamide solution. Even though after polymerization, polyacrylamide is no longer toxic, some unpolymerized acrylamide residue is still present, so gels should always be handled with gloves.

1.3.3. Staining gels with Coomassie Brilliant Blue

For preparing the staining solution, which at the same time is used for fixing the proteins within the gel matrix:

1. Dissolve 0.25% (w/v) Coomassie Brilliant Blue R-250 in ethanol, ddH_2O and glacial acetic acid [5:5:1 (v/v)].
2. Let the solution stir overnight in the dark.
3. Filter the solution
4. Store in a dark bottle

The staining solution can be reused several times, but do not leave it for prolonged time in the staining dish, as this will cause the evaporation of the ethanol and thus reduce the fixation properties of the solution.

Staining of the gel can be done in a glass dish covered with plastic foil or in a household plastic dish with cover:

1. Immerse the gel in the staining solution.
2. Agitate slowly for 16 h (overnight) at room temperature on a slowly rocking platform or orbital shaker.
3. Remove the staining solution and save it for future use. Destain the gel in the same solution [5:5:1 (v/v) of ethanol, ddH_2O and glacial acetic acid] without the dye.
4. Change destaining solution two or three times and continue destaining until blue bands and a clear background are obtained. Inserting a paper tissue into the destaining dish helps to absorb unbound Coomassie Brilliant Blue.
5. Destained gels can be dried in a vacuum gel-drying system; alternatively they can be stored in wet conditions within a sealed plastic container; add a few drops of glycerol to keep the gel soft.

1.3.4. Staining gels with silver salts

Various methods have been developed for staining polypeptides with silver salts after separation by SDS-PAGE, including more or less laborious and more or less sensitive methods. The procedure for silver staining of proteins in polyacrylamide gels described below is based on that described by Blum *et al.* (1987). A polyacrylamide gel with dimensions 100 x 120 x 0.9 mm requires a volume of at least 200 ml of all solutions. The plastic container used should be adapted to the size of the gels to allow complete immersion. Solutions containing thiosulfate have to be prepared freshly to obtain sensitive and reproducible staining results. Powder-free disposable gloves should be worn when handling/transferring gels, as silver stain will detect proteins and oil transferred from fingers.

1. Prepare a fixative solution containing
   1.1. 50% (v/v) methanol.
   1.2. 12% (v/v) acetic acid.
   1.3. 0.5 ml of 37% formaldehyde.
   1.4. Dilute with ddH_2O to 1 l.
2. Incubate gel in the fixing solution for 30 min at room temperature with gentle shaking.
3. Wash for 10 min at room temperature in aqueous solution containing 50% (v/v) ethanol, with gentle shaking.
4. Repeat step 3.
5. Incubate gel for 5 min in a solution containing $Na_2S_2O_3 \cdot 5H_2O$ (0.2 g/l).
6. Rinse gel in water for 20 s.
7. Repeat step 6 twice.
8. Incubate for 10 min at room temperature with gentle shaking in a solution containing:
   8.1. $AgNO_3$ (2 g/l),
   8.2. 37% formaldehyde (0.75 ml/l).
9. Rinse the gel in water for 20 s.
10. Repeat step 9.
11. Add developer solution, made up from:
    11.1. Na_2CO_3 (60 g/l),
    11.2. 37% formaldehyde (0.5 ml/l),
    11.3. $Na_2S_2O_3 \cdot 5H_2O$ (4 mg/l).
12. Incubate gel at room temperature with gentle agitation and carefully watch the developing process.
13. Stop developing process once the desired band intensity and contrast are obtained by adding a solution containing
    13.1. 50% (v/v) methanol.
    13.2. 12% (v/v) acetic acid.
    13.3. 38% ddH_2O.
14. Wait for a few minutes, then wash the gel with ddH_2O.
15. Preserve the gel by drying or in wet condition within a sealed plastic container; add a few drops of glycerol to keep the gel soft.

1.4. Western blotting and immunodetection of proteins separated by SDS-PAGE

Western blotting, also known as immunoblotting, refers to the transfer of proteins from a polyacrylamide gel onto a solid support, such as a nitrocellulose, polyvinylidene difluoride (PVDF), or cationic nylon membrane. This membrane is then used in an immunodetection procedure to reveal specific protein(s).

The transfer of proteins from polyacrylamide gels to membranes

was originally described by Towbin *et al.* (1979). The original method uses a tank containing a large volume of transfer buffer and is referred to as tank blotting. Subsequently, special western blotting systems were developed for semi-dry transfer. These systems are equal in performance, thus preference will depend on already available laboratory equipment.

After transfer, unspecific binding sites of the membrane are first blocked by excess protein added to the incubation buffer to suppress nonspecific adsorption of antibodies. Subsequently, the immobilized proteins are reacted with a specific polyclonal or monoclonal antibody. Antigen-antibody complexes are finally revealed through a secondary antibody and chromogenic or chemiluminescent reactions.

The following protocol uses a tank blotting system and a horseradish peroxidase-conjugated secondary antibody of an enhanced chemoluminescence (ECL) detection system (GE Healthcare) for revealing antigen-antibody complexes:

1. Prepare the western blotting transfer buffer:
   1.1. 25 mM Tris,
   1.2. 192 mM glycine,
   1.3. 20% (v/v) methanol.
2. Cut the PVDF membrane and filter paper sheets to fit the size of the separating gel.
   Be sure to handle the PVDF membrane using powder free gloves and forceps.
3. Activate the membrane in methanol for 1–2 min.
4. Immerse in water for 1 min to remove the activating solvent.
5. Incubate the PVDF membrane in transfer buffer for 2 min.
6. Assemble the blotting sandwich (Fig. 3) in the following order in a tray containing western transfer buffer (make sure to keep all items submersed and avoid including air bubbles, especially so between the gel and the membrane):
   6.1. A stiff plastic supporting grid.
   6.2. A foam sponge or Scotch-Brite pad (3M).
   6.3. Two sheets of thick filter paper.
   6.4. The polyacrylamide gel.
   6.5. The PVDF membrane.
   6.6. Two sheets of thick filter paper.
   6.7. A foam sponge or Scotch-Brite pad (3M).
   6.8. The second stiff plastic supporting grid.
7. Fill the transfer tank with western transfer buffer.
8. Insert the sandwich into the support holder of the blotting apparatus. Make sure the orientation is correct, as transfer is from the cathode (-) to the anode (+), thus, the gel should face towards the cathode and the membrane face the anode.
9. Connect tank to a high voltage power supply.
   This is not your usual electrophoresis power supply, but one that can go up to 200 V, 2000 mA and 200W).
10. Run transfer at a setting of 30 V for about 2.5 h at room temperature.
11. Shut down power supply, disconnect cables.

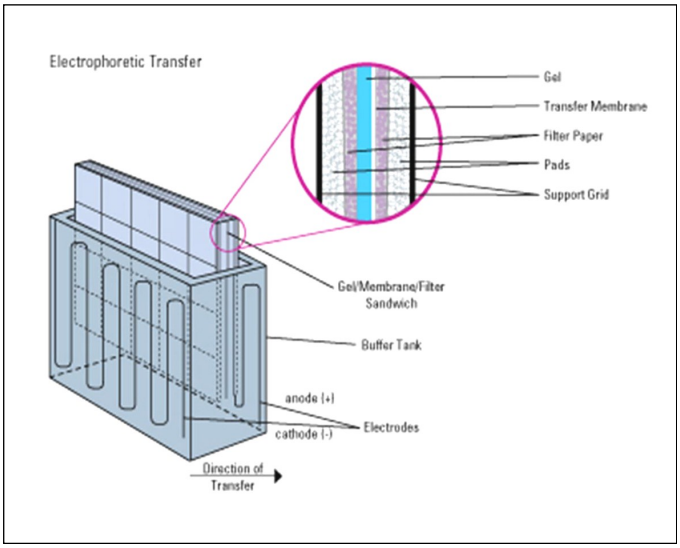

Fig. 3. Setup of gel/membrane blotting sandwich for western blot transfer of proteins. Observe gel/membrane position in the polarity setting [anode (+), cathode (-)].

12. Dismount the blotting sandwich.
13. Mark with a pencil the side of the membrane that faced the gel.

To verify transfer efficiency, the gel can be stained with Coomassie Brilliant Blue after blotting (see section 1.3.3); alternatively, the membrane can be stained with Ponceau S solution [0.5 g of Ponceau S in 100 mL of 1% (v/v) acetic acid aqueous solution] for 2 min, washed two to three times in water and then further destained in water. The PVDF membrane can either be used directly for immunodetection, as described below, or air-dried for later detection (this will require reactivation by immersion in methanol, as described in step 3 of the above list).

For immunodetection:

1. Prepare 1l of a 10 x PBS stock solution:
   1.1. 80 g NaCl,
   1.2. 2 g KCl,
   1.3. 14.4 g Na_2HPO_4,
   1.4. 2.4 g KH_2PO_4,
   1.5. in 1l ddH_2O.
2. Prepare a blocking solution containing 250 ml of buffer A:
   2.1. 3.027 g Tris,
   2.2. 0.147 g $CaCl_2$,
   2.3. 2.33 g NaCl,
   2.4. Adjust pH to 8.5,
   2.5. 50 g non-fat dried milk,
   2.6. Complete the volume to 500 ml with ddH_2O.
3. Block unspecific binding sites by immersing the membrane in this solution for 1 h at room temperature on an orbital shaker. Alternatively, membranes may be left in the blocking solution overnight in a refrigerator.
4. Briefly rinse the membrane with two changes of wash buffer (500 µl Tween 20 in 1l of 1 x PBS (make up from the 10 x stock, described in step 1, and adjust the pH to 7.2).

5. Appropriately dilute the primary antibody in blocking solution. The dilution factor must be determined empirically for each antibody, e.g. through dot blotting of a serial dilution of the antibody.
6. Incubate the membrane in diluted primary antibody for 1 h at room temperature on an orbital shaker.
7. Briefly rinse the membrane with two changes of wash buffer.
8. Keep the membrane in wash buffer for 15 minutes at room temperature.
   Use >4 ml of wash buffer per cm^2 of membrane.
9. Wash the membrane a further three times for 5 min each, in changes of wash buffer.
10. Dilute the horseradish peroxidase (HRP)-conjugated secondary antibody of the ECL kit in wash buffer.
    Again, the dilution factor must be determined empirically for each antibody - a 1:12,000 (v/v) dilution may usually be appropriate.
11. Incubate the membrane in the diluted secondary antibody for 1 h at room temperature on an orbital shaker.
12. Briefly rinse the membrane with two changes of wash buffer.
13. Wash the membrane in > 4 ml/cm^2 of wash buffer for 15 min at room temperature.
14. Wash the membrane a further three times for 5 min each, in changes of wash buffer.
15. Proceed with the detection reaction to obtain the chemoluminescent signal following the manufacturer's instructions. This procedure is specific for each commercial ECL kit.
16. Wrap the blots wetted with ECL solution in household PVC foil and place, protein side up, in an X-ray film cassette.
17. In a dark room place a sheet of autoradiography film on top of the membrane previously wrapped in foil.
18. Close the cassette and expose for a short time, usually 5 min.
19. Immediately develop this first film using commercial X-ray film developer or, if available, an automatic developer system.
20. Based on the obtained band intensity, estimate an optimal exposure time for a second (or third) film.

1.5. Rocket immunoelectrophoresis

Rocket immunoelectrophoresis is a simple, quick and reproducible method for determining the concentration of a single protein in a protein mixture. Like immunodetection following western blotting it is a method based on the affinity of a specific antiserum (which can be mono- or polyclonal) with a specific protein. As it does not use a secondary antibody conjugated with a moiety for high sensitivity detection, but is based on the formation of an antigen-antibody precipitate in a gel matrix. It does not have the sensitivity of the immunodetection method described in section 1.4. It does, however, have the advantages that the presence of a specific protein can be analysed fairly quickly, both qualitatively and quantitatively, in a relatively large number of samples.

In this procedure, appropriately diluted samples are applied to small circular wells cut into an agarose gel which has a specific antibody already incorporated in its matrix. When migrating in an electric field, the protein of interest will eventually reach a critical point of antigen-antibody concentrations resulting in the local formation large precipitating complexes. The agarose gel can then be stained and the rocket-shaped precipitate becomes apparent (Fig. 4). The position of this peak is directly related to the concentration of the protein of interest.

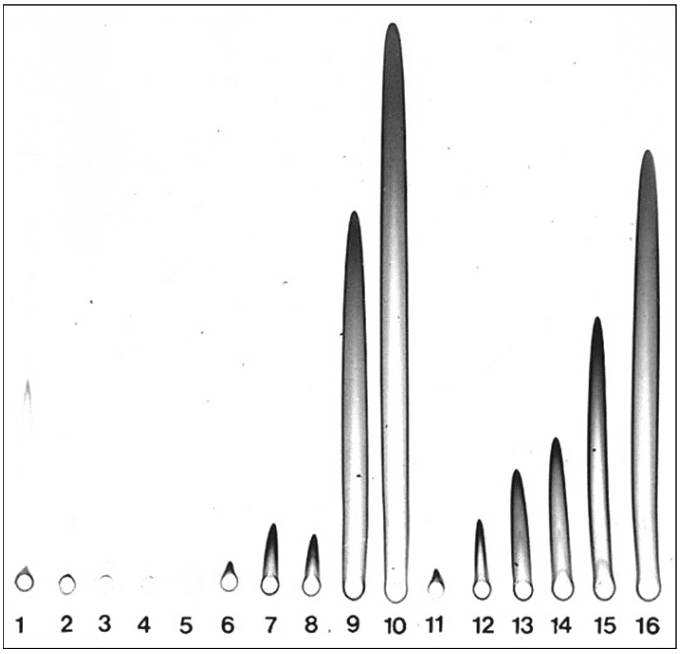

Fig. 4. Rocket immunoeletrophoresis for quantification of haemolymph vitellogenin in 1 to 6 day-old *Apis mellifera* workers reared on different diets. Haemolymph was from bees fed a 0% pollen diet (wells 1-4), a 15% pollen diet (wells 5-7), a 50% pollen diet (wells 8-10), a pollen-free sugar diet supplemented with soybean and yeast (wells 11-13), and naturally fed workers (wells 14-16). Reproduced from Bitondi and Simões (1996). Copyright *Journal of Apicultural Research*

A typical rocket immunoelectrophoresis assay of honey bee haemolymph proteins is done as follows:

1. Prepare a 1% (w/v) agarose solution in 0.06 M Tris-HCl buffer, pH 8.6.
2. Completely dissolve the agarose by boiling for 2-3 min.
3. When the agarose has dissolved, place the flask in a 52ºC water bath.
4. Once the agarose solution has cooled to 52ºC (use a thermometer to check temperature), add an appropriate amount of antiserum (this amount has to be determined empirically by serial dilution assays for each antiserum).

Do not add the antiserum earlier as a higher temperature will cause its denaturation, also do not add it much later, as agarose will soon start to solidify; briefly mix to ensure even dispersal of the antiserum.

5. Pour the agarose solution (15 ml) onto a clean glass plate (usually 10x10 cm) so as to guarantee an even gel thickness (the final gel will be approximately 1.5 mm thick):

5.1. Place the glass plate on a levelled surface.

5.2. Start pouring the agarose solution in the middle of the plate.

The liquid will spread to the edges of the glass plate, but surface tension will prevent it from running off the edge of the plate (alternatively, tape can be used to seal the edges).

6. Wait for 5–10 min for the agarose gel to harden.
7. Using a steel puncher connected to a suction device punch holes of 1 mm diameter at minimal spacing of 0.5 cm forming a line across the plate at approximately 1 cm from one edge. The holes can also be made using a glass Pasteur pipette with a suction bulb.
8. Place the glass plate with the gel on a horizontal electrophoresis system.
9. Fill the troughs with electrode buffer (0.3 M Tris-HCl buffer, pH 8.6).
10. Apply wicks (made from filter paper) immersed in buffer to form a bridge between the gel edges and the buffer troughs. Make sure that the gel is in correct orientation with respect to polarity of the electrophoresis system [the cathode (-) should be next to the sample wells].
11. Add gel buffer (0.06 M Tris-HCl buffer, pH 8.6) at a 1:1 ratio (v/v) to the appropriately diluted samples.
    It is important to ensure that all samples are prepared to an approximately equal volume and, if possible, contain similar amounts of total protein.
12. Apply samples to the well.
    This should be done as quickly as possible to minimize diffusion from the wells into the gel (alternatively a small current of 1-2 mA may be applied during loading to overcome diffusion problems).
13. Immunoelectrophoresis is then carried out at 20ºC for 16 h, at a setting of 0.08 V/cm gel length (make sure to check polarity).
14. For staining after electrophoresis:
    14.1. First, cover the gel with two layers of filter paper soaked in saline 0.9%.
    14.2. Add a 2–3 cm thick layer of dry soft paper tissue.
    14.3. Cover with a thick glass plate to guarantee application of slight and even pressure (about 10 g/cm^2).
15. After 20-30 min the gel layer should have now been reduced to a thin film covering the glass plate.
16. Immerse the gel for 24 h in saline solution.
17. Wash in distilled water for 30 min.
18. Cover the gel with two layers of filter paper soaked in distilled water.
19. Cover with a 2–3 cm thick layer of dry soft tissue paper.
20. Cover with a thick glass plate to apply slight and even pressure (about 10 g/cm^2).
21. Air dry the gel on the glass plate with a hair dryer.
    Hot or cold air can be used.
22. Place gel for 20-30 min in staining solution made up with 0.25% (w/v) Coomassie Brilliant Blue R-250 dissolved in a solution 5:5:1 (v/v) of ethanol, ddH_2O and glacial acetic acid (see section 1.3.3).
23. Remove excess dye by washing the gel in the same solution prepared without the dye.
24. After drying at room temperature the gel can be kept as a permanent record.
25. Measure the peak height of the rocket-like precipitates for each sample.

For absolute quantification compare this to a standard sample for the protein of interest run on the same gel; for relative quantification set the sample with the highest peak as 100%.

1.6. Immunofluorescence detection of proteins in tissue: tubulin localization in ovariole whole mounts as an example of a working protocol

One of the most widely used techniques to study the function and/or localization of proteins is known as immunolabelling or immunolocalization. This is a general technical term that defines the use of specific antibodies to identify the location of molecules or structures within cells or tissues, both in whole mounts and histological sections. Depending on the method of antibody detection, these techniques are divided into two major categories: immunofluorescence, which employs a fluorescence-conjugated secondary antibody, and immunocytochemistry, which uses an enzyme-conjugated secondary antibody and a precipitable enzyme substrate for detection. The choice of an immunolabelling protocol will take into account several factors to obtain reliable staining results, such as the specificity of the antibodies and the general conservation of cell and tissue structure. There are currently no commercially available primary antibodies generated against honey bee proteins. For honey bee research, all antibodies are by definition heterologous ones, having been produced against a protein of another species. Depending on the honey bee protein(s) of interest, these heterologous antibodies can have good cross reactivity, as certain immunoreactive protein domains (epitopes) may be conserved. If available, it is of course preferable to use antibodies produced against specific honey bee proteins (e.g. vitellogenin). When choosing a primary antibody, it is furthermore of interest to note whether it is a polyclonal or monoclonal one. Polyclonal ones were generated by conventional immunization of a laboratory animal, and thus are reactive with several domains (epitopes) of a certain single protein, whereas monoclonal ones were generated by hybridization and subsequent selection for a single epitope.

As far as secondary antibodies are concerned, they must, of course, be directed against the immunoglobulin type of the species in which the primary antibody was produced. Furthermore, secondary antibodies can be whole serum, purified immunoglobulins, or antibody fragments (Fab) corresponding to the antigen binding domain. All such choices will eventually depend on the question to be answered, time investment, prior laboratory experience, and availability of antibodies from commercial or non-commercial suppliers (e.g. the Developmental Studies Hybridoma Bank at the University of Iowa, http://dshb.biology.uiowa.edu, or colleagues).

As it is impractical to provide a comprehensive listing and description of all different immunolocalization techniques and their numerous variants herein, we instead describe in this section a specific protocol as an example for general guidance. This is an immunofluorescence protocol for detecting tubulins in whole-mount ovary preparations of adult honey bees. We considered this as a topic of broader interest, considering the importance of reproductive division of labour between queens and workers of *A. mellifera* as a colony trait. In terms of physiology and biochemistry, this involves, on the one hand, vitellogenin produced and secreted by the fat body, and on the other, the structural organization of the ovariole undergoing oogenesis. It is in the latter process where cytoskeleton proteins play a major role in the differentiation and development of the oocytes and nurse cells, both in adult bees and during postembryonic development (Schmidt-Capella and Hartfelder, 2002; Tanaka and Hartfelder, 2004; Florecki and Hartfelder, 2011).

One of the main steps in the developmental determination of the oocyte and its differentiation within a cluster of cystocytes is the presence of a microtubule-organizing centre or centrosome, which is a structure containing, amongst other proteins, all three members of the tubulin family, α- β- and γ-tubulin. Furthermore, α- and β-tubulin also form heterodimers that make up microtubules, e.g. present in the mitotic or meiotic spindle apparatus. Immunofluorescence detection of α-, β- or γ-tubulin can thus reveal several structures of interest, providing relevant information about cytoskeleton organization and organelle distribution in honey bee oogenesis.

1.6.1. Buffers

1. prepare 100 ml of a 0.02M phosphate-buffered saline (PBS) from the following stock solutions:
   - 1.1. 1.9 ml of 0.2 M $NaH_2PO_4xH_2O$,
   - 1.2. 8.1 ml of 0.2 M $Na_2HPO4xH_2O$,
   - 1.3. 0.9 g NaCl,
   - 1.4. 80 ml ddH_2O,
   - 1.5. Adjust pH to 7.4,
   - 1.6. Complete volume to 100 ml.
2. Prepare a microtubule Stabilization Buffer containing:
   - 2.1. HEPES buffer (25 mM, pH 6.8),
   - 2.2. 25% glycerol,
   - 2.3. 0.5 mM $MgCl_2$,
   - 2.4. 25 µM phenylmethyl sulfonyl fluoride (PMSF),
   - 2.5. 1% Triton X-100,
   - 2.6. 0.01% sodium azide.

   All molarities and % given as final concentrations.

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) is a zwitterionic organic chemical buffering agent. It is widely used in cell culture media for maintaining a physiological pH level. PMSF is a serine protease inhibitor which is best prepared as a stock solution of 25mM PMSF in ethanol. Triton X-100 is a detergent, so it should be added to the buffer only after adjusting the pH. Sodium azide is a common preservative of samples and stock solutions, acting as a bacteriostatic.

1.6.2. Dissection and fixation of ovarioles

1. Dissect the ovaries from adult queens or workers (see the *BEEBOOK* paper on anatomy and dissection (Carreck *et al.*, 2013)).
2. Transfer these to a dish containing honey bee culture medium (recipe and preparation steps as described in Rachinsky and Hartfelder, 1998) or a commercial insect culture medium (e.g. TC- 100).
3. Individualize the ovarioles and, with the aid of fine watchmakers forceps, manually remove the tracheae and the peritoneal sheath covering each ovariole.
4. Transfer batches of individualized clean ovarioles into a 4-well plate containing sufficient (around 0.5 ml) Stabilization Buffer (see 1.6.1., step 2.) to cover the ovarioles.
5. Keep for 20 min at room temperature (RT) under shaking movement.
6. Fix the ovarioles in cold (-20°C) pure methanol for 10 min at 4°C.

Note, the most common fixative used in immunolabelling protocols is 4% paraformaldehyde (PFA) in PBS, but for microtubules, a better result is obtained with methanol fixation following treatment with Stabilization Buffer.

7. Rinse in PBS for 30 min at RT with shaking.
8. Permeabilize in PBS-T (PBS 10 mM, Triton X-100, 0.1 %) for 30 min at RT with shaking.
9. Block unspecific binding sites by incubating the ovarioles in PBS-T supplemented with 10% non-immune serum for 20 min at RT with shaking.

The non-immune serum must correspond to a serum of the organism used as source for generating the secondary antibody, e.g., if using a FITC-conjugated goat-anti-mouse-IgG antibody, use a non-immune goat serum for blocking.

10. Rinse in PBS-T for 2x 15 min at RT with shaking.
11. Incubate for 3 h at RT or at 4°C overnight in a monoclonal antibody raised against α-, β- or γ-tubulin, diluted in PBS-T under shaking.

When using the monoclonal antibody raised against β_{I+II}-tubulin (Sigma-Aldrich, T8538) a dilution of 1:200 in PBS-T may give good results, but such dilutions must be determined empirically by serial dilution experiments for each antibody and tissue.

12. Rinse in PBS-T for 2 x 30 min at RT with shaking.
13. Remove PBS-T.
14. Leave ovarioles for 1 h in a fluorescence-conjugated secondary antibody diluted in PBS-T in darkness at RT under shaking.

In the case of a FITC-conjugated goat-anti-mouse IgG (Sigma-Aldrich), a dilution of 1:400 in PBS-T is sufficient to obtain a good signal to background ratio.

15. Rinse the ovarioles in PBS-T for 2 x 30 min in darkness at RT with shaking.
16. After this wash step one can add TRITC-conjugated phalloidin to label filamentous actin, and/or DAPI (4',6-diamidino-2-phenylindole) or another reagent intercalating with dsDNA to counterstain nuclei.
17. Rinse the ovarioles in PBS-T for 2 x 30 min in darkness at RT with shaking.
18. Rinse briefly in ddH_2O.
19. Transfer ovarioles to microscope slides and embed in glycerol/ n-propyl gallate mounting medium:
    19.1. Glycerol 90%,
    19.2. N-propyl gallate 3% in PBS,
    19.3. Supplemented with sodium azide 0.01%.
    N-propyl gallate is an anti-fade reagent used in fluorescence microscopy to reduce photobleaching; alternatively, you may use commercial antifade reagents (e.g. Vectashield).
20. Coverslip.
21. Seal coverslip edges with nail polish.
21. Store slides in dark.

The whole-mount ovariole preparations can be analysed by conventional epifluorescence microscopy or by means of a laser confocal system.

As in any immunolabelling protocol, a negative control staining is mandatory. In the case of commercial antibodies this can be done by substituting the primary antibody for PBS-T. When using an antibody prepared in the proper laboratory or by a colleague, it is even better to use at this step a pre-immune serum, i.e. serum drawn from the respective animal (usually rabbit) prior to immunization.

User safety: Sodium azide is highly toxic. Appropriate ventilation (laboratory chemical hood) and personal protective equipment (such as gloves) must be used to minimize exposure.

2. Measurement of glucose and trehalose in honey bee haemolymph

This protocol describes a sensitive enzymatic method for quantifying glucose and trehalose in honey bee haemolymph. Haemolymph sugar levels do not only need to be regulated, they also provide important information on the physiological state of carbohydrate metabolism, which is associated with gustatory sucrose responsiveness (Amdam *et al.*, 2006; Wang *et al.*, 2012). In general, the glucose titre in honey bee haemolymph is below 20 µg/µl and the trehalose titer is below 30 µg/µl (Wang *et al.*, 2012).

The method allows measuring of glucose concentrations from 0.5 to 100 µg/µl, and trehalose from 0.4 to 94 µg/µl. Such sensitivity makes the method appropriate for estimation of glucose and trehalose in most insect body fluids, without prior concentration or extraction. Furthermore, these methods are specific, reproducible, sensitive, high throughput and do not require extensive sample preparation. Measuring other carbohydrates of the honey bee colony, such as sugar concentration in honey or the crop of foragers, does not require such a sensitive method and can be easily done by means of a refractometer.

The glucose method described herein is based on the reaction between glucose and adenosine triphosphate (ATP). Glucose can be phosphorylated by adenosine triphosphate (ATP) to form glucose- 6-phosphate (G6P) in a reaction catalysed by hexokinase. G6P is then oxidized to 6-phosphogluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD^+) in a reaction catalysed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD^+ is reduced to NADH, which consequently can be spectophotometrically detected as an increase in absorbance at 340 nm (Peterson and Young, 1968; Bondar *et al.*, 1974). The amount of NAD^+ consumed in the reaction is directly proportional to the amount of glucose present in the sample. For quantifying trehalose, this disaccharide must first be hydrolysed to two molecules of D-glucose in a reaction catalysed by trehalase (Broughton *et al.*, 2005; Flatt and Kawecki, 2007).

2.1 Sample Collection

1. Anesthetize bees by keeping them at 4ºC for a few minutes.
2. Mount them on a wax plate by a pair of insect pins crossing over the waist.
3. Incise dorsally between the 5^{th} and 6^{th} abdominal segment by means of a G 30 needle (BD).
4. Collect 1 µl of haemolymph using a microcapillary.
   Care needs to be taken to avoid contamination from intestine and other surrounding organs.
5. Transfer each haemolymph sample into a 1.5 ml Eppendorf tube.
6. Immediately snap freeze the samples in liquid nitrogen.
7. Store them at -80ºC until testing.

2.2. Preparation of the reagents

2.2.1. Benzoic acid solution

Dissolve 0.1 g of benzoic acid in 100 ml ddH_2O to make a 0.1% solution.

This solution is stable at room temperature.

2.2.2. Glucose standard stock solution (1 mg/ml)

1. Dry the glucose at 60-80ºC for 4 h.
2. Allow to cool in a desiccator.
3. Dissolve 10 g of glucose in benzoic acid solution.
4. Make up to 10 ml in a volumetric flask.

This stock solution is stable for at least six months at 4 ºC; do not freeze.

2.2.3. Enzyme mix solution

The enzyme mix solution contains 1.5 mM NAD^+, 1.0 mM ATP, 1.0 unit/ml of hexokinase, 1.0 unit/ml of glucose-6-phosphate dehydrogenase.

This enzyme mix is commercially available (Sigma-Aldrich) and dissolves readily in ddH_2O. When kept at 4ºC, this solution should be stable for up to one month.

2.3. Glucose assay

1. To make the glucose standard curve, prepare eight clean glass test tubes (5 ml) and label them S1 through S8.
2. Aliquot 0.5, 1, 5, 10, 30, 50 and 100 µl glucose standard solution (1 mg/ml) into tubes S2-S8.

Do not add any standard to tube S1 as this tube will be the blank.

3. Label appropriate number of clean glass test tubes (5 ml) for samples.
4. Keep the tubes on ice.
5. Take the haemolymph samples from the -80ºC freezer and keep them on ice.
6. Add 1 ml enzyme mix solution to each standard tube and sample tube.
7. Invert tubes 4-6 times.
8. Centrifuge tubes for 30 s to spin down contents.
9. Equilibrate reaction mix at room temperature for 15 min.
10. Transfer 200 µl aliquots of each standard (S1-S8) and sample into cuvettes or into a 96-well microplate for spectrophotometry readings.
11. Set spectrophotometer (for cuvettes) or ELISA reader (for microplates) for reading absorbance at 340 nm.

Standards and samples should be read in replicates or triplicates.

12. To calculate glucose concentrations, plot absorbance at 340 nm as a function of glucose concentration of the standard curve samples.

The concentrations of glucose standards (0.5, 1, 5, 10, 30, 50 and 100 µg/ml) are plotted on the X-axis; respective absorbance is on the Y-axis.

13. Determine the equation of the line by linear regression.
14. Each glucose concentration is calculated as:

$$\text{Glucose concentration (ug/ul)} = \frac{[(A_{\text{sample}} - A_{\text{blank}}) - (\text{y-intercept})]}{\text{Slope}}$$

15. If a sample had to be diluted during preparation, the result must be multiplied by the dilution factor, F.

2.4. Trehalose assay

1. After taking the glucose readings, pipette 0.5 µl of the trehalase enzyme into each cuvette or well of the microplate including the wells containing the glucose standards (Flatt *et al.*, 2008; Ishikawa *et al.*, 2009).
2. Slowly shake the plate on a rocking platform for 1 min.
3. Centrifuge for 2 min.
4. After centrifugation, use a piece of Parafilm to seal the cuvettes or microwell plate.
5. Incubate overnight at 37ºC overnight.
6. Centrifuge again.
7. Read absorbance as described above for glucose (step 11 in 2.3)
8. Calculate the trehalose concentration as:

$$\text{Trehalose concentration (ug/ul)} = \frac{\left\{\frac{[(A_{\text{sample}} - A_{\text{blank}}) - (\text{y-intercept})]}{\text{Slope}} - \text{Glucose concentration}\right\} \times 342.3}{180.2 \times 2}$$

The trehalose concentration is calculated as the reading of the proper reaction minus the prior determined glucose concentration. The term is then multiplied by the molecular weight of trehalose (342.3) and, as trehalose is split by trehalase into two glucose molecules, the entire term is finally divided by 2 x the molecular weight of glucose (180.2).

3. Analysis of juvenile hormone and ecdysteroid levels in honey bees

Juvenile hormone (JH) and ecdysteroids are lipid signalling molecules playing fundamental roles in postembryonic development and the reproductive physiology of insects (Nijhout, 1994). In social insects, where these hormones are also involved in regulating caste development, reproductive dominance and division of labour, the role of these hormones has been extensively reviewed (de Wilde and Beetsma, 1976; Nijhout and Wheeler, 1982; Robinson, 1992; Robinson and Vargo, 1997; Hartfelder and Engels, 1998; Hartfelder and Emlen, 2012) ever since their biochemical characterization.

While many of these insights into the roles played by these hormones in social insects have been gained through application of synthetic hormones or hormone analogs, such experiments require confirmation through analyses of circulating hormone titres or rates of hormone synthesis by the respective endocrine glands or peripheral tissues. Conclusions based solely on hormone treatment experiments are frequently and justifiably subject to critique (Zera, 2007) as the applied doses usually exceed endogenous hormone levels by three to six orders of magnitude, thus introducing the possibility of pharmacological effects masking their truly physiological ones. Employing sensitive detection methods is thus paramount to fully understand the role of these lipid signalling molecules.

As a key regulator of insect development, reproduction, and behaviour (Goodman and Cusson, 2012), juvenile hormone (JH) also plays a major role in the social organization of bees, wasps, ants and termites (Hartfelder and Emlen, 2012). In honey bees, JH has been

shown to drive caste development in the larval stages (Hartfelder and Engels, 1998), and in adult workers it plays an important role in division of labour (Robinson and Vargo, 1997; Amdam *et al.*, 2007) and sensory modulation (Pankiw and Page, 2003). There are several isoforms of JH in different insects (e.g. JH I, JH II, JH III, bis-epoxy JH III). These differ slightly in their side chains and unsaturated bonds, but in honey bees, as in most insects, JH III is the only isoform produced by the corpora allata (Hagenguth and Rembold, 1978).

Whereas insects can synthesize JH *de novo* from relatively simple compounds (acetyl CoA or proprionyl CoA) they cannot do this for ecdysteroids. Rather, they require dietary steroids for conversion to physiologically active hormone. This conversion occurs in the prothoracic glands of larvae and pupae and in the gonads of the adults. In honey bees, the predominant ecdysteroid moiety in haemolymph of larvae and pupae and ovaries of adult females is makisterone A, quantitatively followed by 20-hydroxyecdysone and ecdysone (Feldlaufer *et al.*, 1985, 1986; Rachinsky *et al.*, 1990). These are the physiologically active ecdysteroids, whereas others, especially a series of different conjugates, are either metabolites or storage forms (Lafont *et al.*, 2012). In honey bee caste development, haemolymph ecdysteroid titres differ between queen and worker larvae and pupae (Rachinsky *et al.*, 1990; Pinto *et al.*, 2002), but they do not seem to play a major role in reproduction or division of labour (Hartfelder *et al.*, 2002).

With the importance of these hormones in honey bee biology in mind, we will focus in this section primarily on currently used and firmly established analytical methods. We detail radioimmunoassay (RIA) and physicochemical detection methods, such as gas chromatography coupled with mass spectroscopy (GC-MS), for hormone titration, as well as a radiochemical *in vitro* assay for determining the JH-synthetic activity of the *corpora allata* (CA). It is important to note that while radioimmunoassays have frequently been substituted by enzyme-linked immunosorbant assays (ELISAs), due to restrictive regulations for the use of radioisotopes, there are no ELISAs of sufficient sensitivity available for the quantification of insect ecdysteroids and JH.

Older methods, such as the *Galleria* bioassay, will not be described herein. While important tools in the early days of JH quantification, including in honey bees (Fluri *et al.*, 1982), these older methods are extremely laborious, and provide only relative measures (e.g. *Galleria* units) rather than absolute quantities (ng JH per ml haemolymph). We also do not present recently developed analysis methods employing liquid chromatography mass spectrometry (LC-MS) that have been developed (Westerlund and Hoffmann, 2004; Li *et al.*, 2006). Although these have been validated for use in honey bees (Zhou *et al.*, 2011) and are comparable in terms of sensitivity to radioimmunoassays and GC-MS (Chen *et al.*, 2007), they are not yet in common use. A recently developed, very elegant and highly sensitive method for quantifying JH based on tagging the epoxy group of JH with a fluorescent tags, with subsequent analysis by reverse phase high performance liquid chromatography coupled to a fluorescent detector (HPLC-FD) (Rivera-Perez *et al.*, 2012) may, however, eventually become an option. Ultimately, the method of choice for a laboratory will, of course, essentially depend on available equipment and expertise.

3.1. General instructions on haemolymph sample collection and glassware preparation for juvenile hormone assays

3.1.1. Haemolymph collection

The most frequently analysed samples are haemolymph obtained from larvae or adult honey bees. Pupae typically have very low JH titres which are physiologically irrelevant. It is only the pharate adults (pupae undergoing pigmentation of the thorax and abdomen) that may be of interest, as in these stages JH becomes relevant for inducing vitellogenin expression.

1. For collecting haemolymph from feeding-stage larvae:
    1.1. Place the insect on a piece of Parafilm.
    1.2. Identify the position of the dorsal vessel, which is the transparent and easily visible vessel running all along the entire dorsal side of the larva.
    1.3. Puncture this vessel with a pair of forceps.
    1.4. Extruding haemolymph should be clear and can be collect with a microcapillary.
2. For collecting haemolymph from spinning stage larvae and prepupae:
    2.1. Puncture and collect extruding body fluid.

As spinning stage larvae and prepupae are undergoing metamorphosis, the extruding fluid this is not clear haemolymph, but a whitish fluid that contains a lot of tissue debris.

For obtaining the haemolymph fraction:

    2.2. Transfer to a centrifuge tube.
    2.3. Centrifuge at 10,000 x g for 5-10 min.

A thin white sheet containing lipids will have now formed a top layer.

    2.4. Carefully insert a collection capillary tube through this sheet to collect the small volume of clear fluid directly below.

Avoid aspirating the voluminous white bottom layer, which mainly contains cell debris.

3. For collecting haemolymph from pharate adults or adults:
    3.1. Immobilize the bee with two insect pins crosswise inserted into a wax-filled Petri dish and pressing the pins down over the waist.
    3.2. Haemolymph should preferably be collected with a microcapillary from an incision in the neck membrane of adult bees or from the thorax.

This minimizes lipid content in the sample.

When collecting haemolymph, a graduated microcapillary tube should be used to ensure accurate assessment of volume. For both GC-MS and RIA, 2 - 4 µl are normally sufficient for a sample. Depending on the stage being sampled, it may be necessary to pool haemolymph from multiple individuals to get readable results. After registering the exact volume of haemolymph, this is expelled into a glass collection vial already containing appropriate solvent.

The vials should be glass, fitted with a screw cap containing a Teflon-lined rubber septum. The Teflon cover should face the sample. These are low-cost glass vials customarily used for GC analysis. For JH analysis by RIA, vials of 1.25-2 ml volume containing 0.5 ml acetonitrile are recommended. For JH analysis by GC-MS, the samples should be collected into 8 ml vials containing 1.5 ml of 50% acetonitrile in water. Samples can then be stored for long periods of time at -20°C. Special refrigeration on transport for short periods (up to two days) is not required.

Do not use plastic vials, such as Eppendorf tubes, to store samples, as JH binds to plastic. It is also not recommended to store haemolymph in microcapillaries in the freezer, as JH degrading enzymes may retain activity under such conditions.

3.1.2. Glassware preparation

JH is a "sticky" lipophilic molecule which makes the use of clean glassware an imperative component throughout all steps of sample preparation. Furthermore, organic solvents can extract compounds from plastic vials, thus preventing accurate assessment of JH, especially when using a GC-MS protocol. Thus:

- Do not use plastic vials at any step and use disposable glassware whenever possible.
- Do not mix glassware used in the preparation of stock solutions from commercial JH with those used in the preparation of biological samples.
- It is strongly recommended that glassware be extensively cleaned and heated to remove contaminants prior to use, especially if the glassware is being reused.

The following procedures for treating glassware are recommended.

3.1.2.1. For GC-MS analysis

1. Clean all glassware by washing in acetone 3 times.
2. Wash in hexane 3 times.
3. Bake overnight at 150°C.

3.1.2.2. For JH-RIA

1. Wash non-disposable glassware for re-use extensively with ethanol, preferably in a sonicating bath.
2. Wash in soapy water.
3. Rinse thoroughly with distilled water.
4. Incubate overnight in 1 M HCl.
5. Rinse in water.
6. Neutralize in 1 M NaOH for 1 h.
7. Rinse thoroughly in distilled water.
8. Dry and heat for 2 days at 150-250 ^{o}C.

3.2. Juvenile hormone extraction, purification and quantification by GC-MS

JH has principally been quantified using one of two procedures, radioimmunoassay (RIA) or gas chromatography-mass spectrometry (GC-MS), where mass spectrometer is the quadrupole mass spectrometer, herein referred to by the trade name "Mass Selective Detector" (MSD). Generally, the GC-MSD approach purifies extracted JH III, then converts it to a d3-methoxyhydrin derivative prior to quantification. This technique was initially developed by Bergot *et al*. (1981), modified by Shu *et al*. (1997), and adapted for use in honey bees by Amdam *et al*. (2010). For further protocol details on GC-MS applications in honey bee research see the *BEEBOOK* paper on chemical ecology (Torto *et al.,* 2013).

The major advantage of this protocol is its high resolution, providing the capacity to quantify significant differences between relatively small quantities of the hormone. Its major limitations are the time necessary to process samples, its relatively high cost, and having to maintain sensitive equipment.

3.2.1. JH sample purification and quantification by GC-MSD

1. Clean all glassware by washing in acetone and hexane (3 times each) then baking overnight at 150°C (see section 3.1.1) prior to use.
2. Prepare solutions of:
   2.1. Ethyl ether: hexane 10:90 (v/v).
   2.2. Ethyl ether: hexane 30:70 (v/v).
   2.3. Ethyl acetate: hexane 50:50 (v/v).

   All solvents should be HPLC grade.
   2.4. Store at -20 °C until use.
3. Prepare a labelled 8 ml borosilicate glass vial with a Teflon lined cap for each sample.
4. Add 1.5 ml of 50% aqueous acetonitrile to each vial.
5. Dilute 200 pg of farnesol (Sigma-Aldrich) in 10 µl hexane. Farnesol will serve as an internal standard.
6. Add farnesol to the vials prepared at step 4.
7. After collecting a 2-4 µl sample of haemolymph (see section 3.1.1), expel it into the prepared sample vial.
8. Prepare a positive control vial, containing 200 pg JH III (Sigma-Aldrich) in 0.5 ml of 50% aqueous acetonitrile.
9. Prepare a negative control vial with just 50% aqueous acetonitrile.
10. Add 2.5 ml hexane to each sample, using a graduated glass pipette.
11. Mix thoroughly with a vortexer.
12. The JH should partition into the upper hexane layer. If the layers fail to separate well, centrifuge the vials at 1000 rpm for 1 min.

13. Using a flint glass Pasteur pipette, remove the hexane layer and transfer it into a fresh 8-ml vial.
14. Repeat the partitioning process twice, adding 2.5 ml hexane each time, for a total volume of 7.5 ml hexane extract.
15. Discard the bottom layer after the third extraction step.
16. Dry the hexane extracts completely by vacuum centrifugation or under a nitrogen stream (UHP grade).
17. While the samples are drying, prepare glass columns under a laboratory hood:
    17.1. Insert a small plug of glass wool at the narrow end of a Pasteur pipette that is sufficient to plug the column, but not so tightly packed as to impede the solvent flow rate.
    17.2. Place the pipettes in the holes of a column holder with a drip tray underneath.
    17.3. Add water [6% (v/w)] to Al_2O_3 powder (activated, neutral, Brockmann I; Sigma-Aldrich).
    17.4. Mix until completely dry.
    17.5. Add 2 ml of the activated Al_2O_3 to the columns.
    17.6. Add 750 µl of hexane to the columns

If the columns drip, add activated Al_2O_3 until they hold the volume.

    17.7. Wash the columns twice with 900 µl of hexane, allowing the hexane to drip into the tray.
18. Add 300 µl of hexane to each sample vial.
19. Cap.
20. Mix thoroughly to dissolve JH from the vessel walls.
21. Transfer each JH sample to a column, using clean glass pipettes.
22. Wash each sample vial twice more, adding 300 µl of hexane each time, thus transferring a total volume of 900 µl per sample to each column.
23. Add another 900 µl of hexane to each column to remove any remaining nonpolar compounds.
24. Wash each column twice with 900 µl of ethyl ether: hexane (10:90), allowing the flow-through to pass into the drip tray.
25. Wash each column with 750 µl of ethyl ether: hexane (30:70), again allowing the flow-through to drip into the tray.
26. Elute each column with 900 µl of ethyl ether: hexane (30:70) and collect the flow-through in new 8 ml vials.
27. Repeat step 26 once and pool both eluates in the same vial, for a total volume of 1.8 ml.
28. Discard the columns.
29. Concentrate the samples to dryness by vacuum centrifugation (~15 min) or under a nitrogen stream.
30. While drying the samples, equilibrate the ampoules containing the methanol-d_4 to room temperature.

Methanol-d_4 can absorb water from air, which may quench the reaction; raising the ampoules' temperature reduces the likelihood of condensation forming.

31. With a micropipettor slowly add 75 µl of methanol-d_4 to each sample vial.
32. Using a micropipettor, add 53 µl of trifluoroacetic acid (TFA) (spectrophotometric grade) to a 1 ml methanol-d_4 ampoule.
33. Mix gently to make enough 5% trifluoroacetic acid:methanol-d_4 solution for 12 samples.
    When doing so, evacuate air from the TFA container with nitrogen before storage.
34. Slowly add 75 µl of 5% trifluoroacetic acid:methanol-d_4 to each sample.
35. Tightly cap the vials and gently mix the contents.
36. Heat the sample vials for 20 min at 60°C to produce d3-methoxyhydrin derivatives.
37. Prepare new columns as described above (step 17).
38. Remove the samples from the oven (step 36).
39. Add 500 µl of hexane to each vial.
40. Mix well to remove residue from the walls.
41. Concentrate the samples to dryness by vacuum centrifugation (~15 min) or under a nitrogen stream.
42. Add 300 µl of hexane to each vial containing dried extract.
43. Mix thoroughly.
44. Transfer each sample to a column using glass pipettes.
45. Rinse sample vial twice, adding 300 µl of hexane each time, thus transferring a total volume of 900 µl to each column.
46. Wash each column twice with 900 µl of ethyl ether: hexane (30:70), allowing the flow-through to drip into the tray.
47. Wash each column twice with 750 µl of ethyl acetate: hexane (50:50), allowing the flow-through to drip into the tray.
48. Add 900 µl of ethyl ether: hexane (50:50) to each column and collect the flow-through in new 8-ml vials.
49. Repeat step 48 once, pooling both eluates in the same vial, for a total volume of 1.8 ml.
50. Discard the columns.
51. Concentrate the samples to dryness by vacuum centrifugation (~15 min) or under a nitrogen stream.
52. Add 300 µl of hexane to each sample and mix well to resuspend JH.
53. Evaporate the hexane under nitrogen gas (UHP grade) to ~25 µl of liquid.
54. Use a glass pipette to transfer the fluid into a tapered 250 µl glass vial insert (e.g. Agilent #5181-1270).
55. Repeat the steps 50-52 for obtaining a final concentrate volume of ~50 µl.
56. Using nitrogen, dry the liquid in the vial insert down to a final volume of 3 µl, measured with a graduated 10 µl syringe.
57. Using the syringe, repeatedly wash down the sides of the insert during the drying process to ensure all JH is within the remaining concentrate.

Repeated flushing through the syringe will also help evaporate down the last few microliters.

58. Syringes should be washed after each use by drawing up acetone then hexane (at least 5 times each).

59. Prepare the GC-MSD system for analysing samples:
    59.1. Install a Zebron ZB-Wax 30 m x 0.25 mm x 0.25 µm GC capillary column.
    59.2. Set initial temperature of GC to hold for 1 min at 60°C.
    59.3. Ramp the GC temperature up at 20°C / min to a final temperature of 240°C; hold for 20 min (i.e. a total run time of 30 min).
    59.4. Set the GC inlet protocol to pulsed splitless injection at 250°C.
    59.5. Set the pressure at 9.98 psi with a 23.8 ml/min flow rate for the carrier gas (UHP grade helium).
    59.6. Set the injection quantity to 1µl.
    59.7. Create a 5-min solvent delay.

    This prevents the recording of highly volatile solvents, which evaporate early on and can damage the detector.
60. Manually load the 1 µl of the concentrated sample using an injection syringe approved by the GC-MSD manufacturer.
61. To ensure accuracy, first draw up 1-2 µl of air, then the sample then another 1-2 µl of air.
62. To ensure specificity, monitor MSD results at m/x 76 and 225 for the JH-III derivative, and at 69, 84 and 136 for farnesol.
63. Measure the peak area for JH and adjust the value to account for any changes indicated by the results for farnesol, which was added in step 6 to serve as internal standard
64. Calculate the final titre based on the haemolymph volume and a standard curve.

    The latter can be prepared with 5, 25, 125, 250 µg or more JH III, and starting the process at step 30.

3.3. Juvenile hormone quantification by radioimmunoassay

Functionally, radioimmunoassays are competition assays, whereby a radiolabelled ligand competes with equivalent nonradioactive moieties from a sample for antigen binding sites of a highly specific antibody. The limiting factor in such assays is always the concentration of the antibody for which the ligands compete. Thus, to be efficient and sensitive, antibody concentrations must be chosen so as to allow a maximal binding ratio of less than 50% for the radioactive ligand, in a standard solution that is free of unlabelled ligand. Once this competition for antibody binding sites has reached an equilibrium, antibody-complexed antigen is separated from the remaining unbound antigen, either chemically, generally by ammonium sulphate precipitation, or immunologically, by means of a secondary antibody or Protein A. Radioactivity in the resultant precipitate is then counted by scintillation spectrometry. The isotopes most frequently used for labelling antigens are ^{3}H or ^{125}J. Since the latter is suitable only for labelling proteins or peptides, all juvenile hormone and ecdysteroid radioimmunoassays use tritiated (^{3}H) compounds.

An in-depth discussion on the radioimmunoassay for juvenile hormones has been provided by Granger and Goodman (1988), including a very detailed description on antiserum production, which will not be addressed here, as this is a rather complicated process and, once a suitable antibody has been generated, it is usually shared within the community. Sharing suitable antibodies has the further advantage that assays are easily comparable among laboratories that run such assays.

Hence, the protocol described here uses a specific antiserum produced by conjugation of JH III to thyroglobulin (Goodman, 1990) and injection into rabbits. The general radioimmunoassay protocol for the detection of JH by means of this antiserum has originally been given by Goodman *et al.* (1990) and subsequently, in a slightly modified version, by Goodman *et al.* (1995). We have used this protocol and adapted it for use with honey bees (Guidugli *et al.*, 2005; Amdam *et al.*, 2007; Marco Antonio *et al.*, 2008). As JH III is the only juvenile hormone homolog present in honey bees (Trautmann *et al.*, 1974), just as in most other insect orders, this greatly facilitates data analysis.

The method described here is certainly not the first serum and RIA protocol used for the quantification of JH titres in honey bees. A highly sensitive but slightly more laborious protocol using a different antiserum and an assay based on equilibrium dialysis for detection of insect JHs has been developed by Strambi *et al.* (1981) and applied to honey bee larvae (Rachinsky *et al.*, 1990) and adults (Robinson *et al.*, 1987), and a direct comparison using the two radioimmunoassay on honey bee samples has validated both assays as equally sensitive (Goodman *et al.*, 1993). A third RIA, with an enantiomere-specific antiserum developed by Hunnicut *et al.* (1989) has also been used on honey bee samples, primarily in the context of division of labour in workers (Huang *et al.*, 1994; Jassim *et al.*, 2000). Finally, it is worthy of note that there are no commercially available JH antibodies.

The following protocol, which is the currently most frequently run, uses an antibody developed by Walter G Goodman (Univ. Wisconsin, Madison). It is divided into four parts: sample preparation and JH extraction, preparation of radioimmunoassay solutions and assay standardization, running the assay, and data analysis.

3.3.1. JH extraction

1. Transfer the entire acetonitrile sample into a 12 x 75 mm disposable glass test tube, but avoid transferring debris that may have accumulated during storage.
2. Add 1 ml of 0.9% NaCl.
3. Add 1 ml of hexane.
4. Vortex thoroughly for 10 s.
5. Keep vials on ice for 30 min.
6. Centrifuge for 5 min at low speed for complete phase separation.
7. Transfer the hexane phase (upper layer) to a new 12 x 75 mm glass test tube.
8. Repeat steps 2 to 7 twice, each time adding 1 ml of hexane to the acetonitrile/saline phase.

9. Pool the hexane phases of each extraction step.
10. Evaporate hexane to dryness by vacuum centrifugation or under an N_2-stream.
11. Re-dissolve JH in 40 µl of toluene containing 0.5% propanediol.
12. Transfer liquid to a RIA glass vial (disposable 6-8 x 40-50 mm test tubes).
13. Wash the extraction vial with another 40 µl of toluene/propanediol.
14. Add to RIA vial (step 12).

3.3.2. Preparation of RIA solutions

3.3.2.1. JH-III standard

As commercial JH III (Sigma or other supplier) is usually not sufficiently pure and there is degradation over time, it is recommended that an aliquot of commercial JH III is first purified by thin layer chromatography (TLC). To do so:

1. "Wash" a glass backed TLC plate (5x20 cm or 20x20 cm, Merck silica gel 60 F_{254}) in ethanol. This is done by placing it in a TLC glass tank with 1 cm of ethanol in the bottom; when the solvent reaches the top of the plate, remove and dry it; it may then be stored.
2. Prior to the actual separation, put a washed TLC a plate into a tank containing a 1 cm high volume of toluene: ethylacetate (95:5) solvent mix and run it in the tank until it reaches the top of the plate. This will clean and activate the plate.
3. Mark the top to indicate plate orientation.
4. Dry the plate.
5. Activate it by heat treatment at 60°C for 10 min.
6. On the bottom side, using a soft pencil, draw a line across at 1.5 cm.
7. At the sides, use the edge of a fine spatula to scratch a line at 0.5 cm from each edge to prevent edge migration distortion.
8. Streak an aliquot of the commercial JH III on the 1.5 cm bottom line.
9. Completely evaporate solvent with an air stream.
10. Run the plate in toluene: ethylacetate (95:5) solvent mix until the solvent front has risen approximately 5 cm into the plate.
11. Remove, air dry and return the plate to the solvent to complete the run to about 17 cm of the total plate length.
12. Air dry the plate.
13. Visualize the JH band under UV light (254 nm).
14. Mark its position with a pencil.
15. Scratch the silica layer corresponding to the JH band from the plate using a spatula, collecting the scraps on a piece of aluminium foil.
16. Transfer scraps to a screw cap glass vial containing 5 ml of hexane/ethyl acetate 85:15.
17. Agitate on an orbital shaker overnight.
18. Filter solvent through a glass syringe fitted with a glass filter.
19. Dry the solvent by vacuum centrifugation.
20. Re-dissolve in toluene containing 0.5% propanediole (final conc.). This will be the purified JH solution for storage at -20°C.

For preparing the JH-III standard for preparation of standard curves:

1. Take a 5 µl aliquot of this purified JH.
2. Dilute 1:100 in methanol.
3. Quantify JH-III by UV spectroscopy at λ = 217 nm. At this wavelength an absorption of 0.502 corresponds to a JH III concentration of 10 µg/ml.
4. Correcting for dilution (step 2), dilute an appropriate aliquot of the purified JH in toluene/propanediol to a final concentration of 50 pg/µl. This will be the stock solution for preparing standard curves.

3.3.2.2. Phosphate buffer

Prepare a 0.1 M phosphate buffer (pH 7.2-7.4) from 0.2 M mono- and dibasic potassium phosphate stock solutions and add sodium azide to a 0.02% final concentration.

3.3.2.3. Saturated ammonium sulphate

1. Add 80 g of ammonium sulphate to 100 ml warm distilled water.
2. Bring to a boil.
3. Make sure that nearly all ammonium sulphate is dissolved.
4. Let cool to room temperature and observe the formation of crystals.
5. Transfer to screw top bottle and store in refrigerator, more crystals will form at the bottom.
6. For use, draw from supernatant without disturbing the crystal bed.

3.3.2.4. Solution of radioactive JH (RIA tracer solution)

The stability of JH-III in aqueous solution is limited. As this solution should be used within 4-6 weeks, it is necessary to estimate the volume needed for each assay series. 100 µl of this solution is required per sample, and the radioactivity should be 6,000-6,500 cpm/100 µl tritiated juvenile hormone III ([10-^{3}H(N)] Juvenile hormone III, Perkin Elmer NET586050UC).

1. Tritiated juvenile hormone III comes as 50 µCi in 0.5 ml toluene/ hexane and an appropriate volume to be used in an assay series must first be evaporated to dryness in a glass test tube.
2. Re-dissolve the dry radioactive JH in the appropriate volume of phosphate buffer (see section 3.3.2.2).
3. Transfer to a screw cap glass vial.
4. Keep vial on orbital shaker for 24 h at 4°C.
5. Test radioactivity by liquid scintillation counting of 100 µl aliquots.
6. The level of radioactivity should be 6,000-6,500 cpm/100 µl. If necessary adjust by adding phosphate buffer or tracer.

3.3.2.5. Antibody solution

Depending on the number of samples to be processed, prepare an adequate volume of RIA serum, Typically, a minimum of 10 ml solution should be prepared, which would be sufficient for 100 samples (100 µl of this solution are required per sample). The solution should be stored at 4°C and should also be used within 4-6 weeks.

For making 10 ml of antibody solution:

1. Dissolve 10 mg of bovine serum albumin (BSA, Fraction V)) and 10 mg of IgG from rabbit serum (Sigma I5006) in 10 ml of 0.1 M phosphate buffer (3.3.2.2).
2. Remove a 0.5-1 ml aliquot of this solution to later be used with the non-specific binding (background) tubes in the assay.
3. Add an appropriate volume of the JH antibody to the remaining RIA serum solution.

Note: The appropriate concentration of JH antibody will have to be established in prior tests evaluating sensitivity through a dilution series of the respective antibody, remembering that an RIA is an isotope dilution assay whereby the specific antibody is the limiting factor. Ideal antibody concentrations should give maximal-binding cpm values corresponding to 35-40% of the total radioactivity added to each sample (around 2000-2500 cpm under the above described conditions). Such tests also need to be run periodically to ascertain binding and, if necessary, adjust the antibody concentration.

3.3.3. Running a JH RIA

1. To set up a standard curve (in duplicates), prepare 10 disposable borosilicate glass culture tubes (6 x 50 mm).

Eight of each series will receive aliquots of 0.5, 1, 2, 5, 10, 20, 50 and 100 µl (these corresponding to 25, 50, 100, 250, 500, 1000, 2500 and 5000 pg of JH- III) of the stock solution of unlabelled JH-III (see section 3.3.2.1).

Note that pipetting of the standard curve samples must be done with Hamilton-type syringes that are exclusively dedicated to use in this JH RIA.

2. The additional four tubes will not receive any unlabelled JH. One of each series will receive antibody-free background solution (to assess non-specific binding) and the other receives radiolabelled JH only (maximum binding values).
3. Evaporate the solvent from all RIA vials, both the standard curve and the sample tubes.
4. Add 100 µl of the RIA tracer solution (3.3.2.4) to each vial.
5. Vortex vigorously for 10 s.
6. Add 100 µl of the antibody solution (3.3.2.5) to all tubes, except the two for background counting, which will receive an equal amount of the background protein solution (3.3.2.5. step 2).
7. Gently mix the tracer with the serum components. Do not vortex as this will cause bubbles to form.
8. Quickly (30 s) spin the tubes in a centrifuge (2000 x g) to remove any solution adhering to the walls of the vials.
9. Cover RIA test tubes with an adhesive sheet to avoid loss by evaporation.
10. Incubate samples overnight at 4°C to attain a binding equilibrium. Do not incubate for more than 24 h.
11. Add 200 µl of saturated ammonium sulphate solution (see section 3.3.2.3) to the test tubes to precipitate proteins, including the antibody-bound JH.
12. Vortex.
13. Store samples at 4°C for 30 min.
14. Centrifuge at 7,500 x g for 15 min at 4°C.
15. Remove supernatant, taking care to leave the pellet undisturbed.
16. Add 400 µL of a 50% ammonium sulphate solution [1:1 saturated ammonium sulphate/water (v:v)] to the test tubes to dissolve the pellet.
17. Vortex.
18. Store samples at 4°C for 30 min.
19. Centrifuge at 7,500 x g for 15 min at 4°C.
20. Remove supernatant, taking care to leave the pellet undisturbed.
21. Add 50 µl of water to the pellet.
22. Vortex to dissolve completely.
23. Transfer the dissolved precipitate to a 20 ml vial appropriate for liquid scintillation counting (LSC) vial.
24. Wash the RIA test tube with another 50 µl of water and add to the same respective LSC vial.
25. Add 5 ml of a biodegradable LSC cocktail (e.g. Optiphase Hisafe3, Perkin-Elmer).
26. Leave the counting vials for 1 h at room temperature before starting the counting process in a β-counter.
27. Counting times should be set to allow a 5 sigma level of confidence.

This period will vary depending on the number of counts in the sample, counting efficiency and instrument variables. Most LSC instrumentation includes variable counting to provide 5 sigma margins of error in their programs. As a rule of thumb for counting the RIA samples, counting times of 2 min should be sufficient, and the entire series should be counted at least twice

3.3.4. Data analysis

The use of the ImmunoAssay Calculations spreadsheet freely available from Bachem (http://www.bachem.com/service-support/immunoassay-calculator) is highly recommended. This program is designed to run a four parameter non-linear regression analysis of RIA data.

In this spreadsheet the mean of the non-specific binding counts (NSB) is first subtracted from the means of all other samples. Next, each of the standard curve means (B-NSB) is divided by the maximum count (B-NSB/B_0-NSB) and these values are then fitted to a nonlinear regression curve given as:

$$y = ((a-d)/(1 + (x/c)^b))+d$$

$$x = c\,((y-a)/(d-y))^{(1/b)}$$

where *a* represents the maximum binding value (set to 1), *b* the slope, *c* the inflection point of the curve (IC_{50}) and *d* the minimum value. Through a graphic conversion of the minimization procedure, the spreadsheet makes this fitting a user friendly task. Finally, sample values are entered and converted to amounts of hormone in each sample.

Such non-linear regression is far superior over a linear log/logit regression analysis for calculating hormone concentrations in samples close to the detection limit of the RIA.

An important quality check for all radioimmunoassays is the calculation of intra- and inter-assay variation. Whereas intra-assay variation usually reflects the level of pipetting errors, inter-assay variation is of relevance when large sample series are processed or when results are to be compared to other studies. This is done by including in each assay a sample prepared from an aliquot of a large haemolymph-pool sample. As this sample will be included in any of the other assays done on the same species, it allows for quality control of the RIA and to assess inter-assay variation.

3.3.5. User safety

The use of radioactive compounds ([10-^{3}H(N)] Juvenile hormone III) requires authorization for individual user and laboratory according to national regulations and guidelines. Working with gloves is required to avoid direct contact with skin, but no further protection from radiation is necessary due to the low energy of tritiated compounds. Disposable material (glass) used during handling of radioactive compounds and liquid residues, as well as scintillation cocktail containing radioactivity must all be properly disposed (follow national guidelines). Toluene and ethyl acetate are flammable solvents. UV-light (254 nm) causes DNA damage and is especially harmful to the eyes; use protective glasses or a UV shield.

3.4. Quantification of ecdysteroids by radioimmunoassay

As far as ecdysteroid quantification is concerned, the same general principles described for the juvenile hormone RIA also apply to the detection of ecdysteroids, except that not a single but several ecdysteroids may naturally be present in haemolymph or tissues. For this reason, when ecdysteroids are not fractionated and separated by specific chromatography methods prior to radioimmunoassaying, results are given as immunoreactive ecdysteroids in relation to the compound used to set up the standard curve, which is usually 20-hydroxyecdysone.

Basically two ecdysteroid RIAs have been used in honey bees, one developed by de Reggi *et al.* (1975), in a protocol using equilibrium dialysis to quantify larval and pupal and adult ecdysteroids (Rachinsky *et al.*, 1990; Robinson *et al.*, 1991), and the other based on an antiserum and protocol developed by Bollenbacher *et al.* (1983). The latter was used more frequently for studies on honey bee ecdysteroids (Feldlaufer and Hartfelder, 1997; Feldlaufer *et al.*, 1985, 1986; Hartfelder *et al.*, 2002; Pinto *et al.*, 2002; Amdam *et al.*, 2004; Nascimento *et al.*, 2004). It is worthy of note that there are no commercially available ecdysone antibodies.

The following protocol uses an antibody developed in the Gilbert laboratory (Univ. North Carolina at Chapel Hill) against a hemisuccinate derivative of ecdysone (Bollenbacher *et al.*, 1983; Warren and Gilbert, 1986). Tritiated ecdysone serves as the labelled ligand and standard curves are established with 20-hydroxyecdysone (20E) as the non-radioactive ligand. Results are, therefore, expressed as 20E equivalents. The protocol is essentially divided into four parts: sample preparation, preparation of radioimmunoassay solutions and assay standardization, running the assay, and data analysis.

3.4.1. Sample preparation

3.4.1.1. for haemolymph

1. Collect the haemolymph into a calibrated microcapillary as described for the JH radioimmunoassay (see section 3.1.1).
2. Note its exact volume.
3. Transfer the haemolymph into an Eppendorf tube containing cold methanol in a 1:100 volume ratio (100 µl methanol per µl haemolymph).
4. Keep at 4°C for a couple of hours (best overnight).
5. Samples can be stored at -20°C in these Eppendorf tubes, as ecdysteroids will not adhere to plastic the way JH does.
6. Centrifuge the tubes to pelletize any remaining impurities.

Most haemolymph samples can be assayed directly by RIA. If there are problems, try purifying free ecdysteroids by chromatographic separation (see section 3.4.1.3., below).

3.4.1.2. for tissue

1. Weigh tissue.
2. Homogenize in at least 500x (w:v) cold methanol.
3. Keep at 4°C for a couple of hours (best overnight).
4. Centrifuge to pelletize and remove impurities.
5. Store supernatant at -20°C.

3.4.1.3. in case of lipid-rich samples (e.g. whole larva)

An additional purification step is usually required as lipids may interfere in the ligand-binding and the ammonium sulphate precipitation of the antigen-antibody complex. A rapid and efficient purification is the separation of free ecdysteroids from polar or apolar compounds by means of reverse-phase liquid chromatography on disposable reverse-phase columns (SepPak C_{18}, Waters, Waters, WAT051910).

1. Evaporate methanol from sample (after centrifugation to remove impurities) by vacuum centrifugation.
2. Resuspend sample in 30% methanol.
3. Activate SepPak C_{18} cartridge mounted on a 1 ml syringe by slowly passing pure methanol.
4. Equilibrate column by passing 1 ml 30% methanol.
5. Load sample on column
6. Pass 2 x 1 ml 30% methanol.

This eluate contains polar ecdysteroid conjugates that can be discarded, or if of interest, analysed after enzymatic digestion (*Helix pomatia* juice).

7. Pass 2 x 1 ml 60% methanol through column and collect eluate in a 5 ml disposable glass test tube.

This eluate contains the free ecdysteroids

8. Discard column.
9. Evaporate solvent from the free ecdysteroid fraction samples by vacuum centrifugation.
10. Re-dissolve in 100% methanol for the RIA procedure.

3.4.2. Preparation of RIA solutions

3.4.2.1. 20-hydroxyecdysone (20E) standard

Prepare a stock solution of unlabelled 20-hydroxyecdysone (20E) from a commercial source (Sigma) diluted to a concentration of 50 pg/µl in methanol. As commercial ecdysteroids usually are not sufficiently pure and there is degradation over time, it is recommended that an aliquot of commercial 20E is first purified by thin layer chromatography (TLC) or, if available by HPLC. The steps for TLC purification are essentially the same ones as described for cleaning up commercial JH (see section 3.3.2.1), the only differences are:

- the solvent mix used for the chromatography run, which is dichloromethane : methanol (85:15) in steps 2 and 10.
- the solvent used to elute TLC-separated 20E from the silica scraps is methanol (step 16).

For preparing the 20E standard for preparation of standard curves:

1. Take a 5 µl aliquot of this purified 20E and dilute 1:100 in methanol for quantification of 20E by spectroscopy at λ = 245 nm.
2. Calculate the 20E concentration in mg/ml as: (0.48 x absorption x dilution factor)/12.6.
3. Correcting for dilution, dilute an appropriate aliquot of the purified 20E in methanol to a final concentration of 50 pg/µl.

3.4.2.2. Phosphate buffer (this is the same as in the JH RIA, see section 3.3.2.2.)

3.4.2.3. Saturated ammonium sulphate (this is the same as in the JH RIA, see section 3.3.2.3.)

3.4.2.4. Solution of radioactive ecdysone (RIA tracer solution)

The stability of ecdysone in aqueous solution is limited. As this solution should be used within 4-6 weeks, it is necessary to estimate the volume needed for each assay series. 100 µl of this solution is required per sample, and the radioactivity should be 5,000-6,000 cpm/100 µl tritiated ecdysone ([23,24-3H(N)]ecdysone, Perkin Elmer NEN; NET621050UC; 1.85 MBq; 1.85-4.07 TBq/mmol). The solution should be stored at 4°C.

1. As tritiated ecdysone comes as 50 µCi in 0.5 ml ethanol, calculate the volume to be used in an assay series.
2. Transfer to 5 ml disposable glass test tube and evaporated to dryness by vacuum centrifugation or under a N_2-stream.
3. Dissolve the radioactive ecdysone in the appropriate volume of 0.1 M phosphate buffer.
4. Transfer to a screw cap glass vial.
5. Keep this vial on an orbital shaker for 24 h at 4°C.
6. Test radioactivity level by liquid scintillation counting of 100 µl aliquots.

This should give 5,000-6,000 cpm for 100 µl.

7. If necessary, adjust radioactivity level by adding phosphate buffer or tracer.

3.4.2.5. Antibody solution (RIA serum)

Prepare an adequate volume of RIA serum for the number of samples to be run. Typically, a minimum of 10 ml solution should be prepared, which would be sufficient for 100 samples (100 µl of this solution are required per sample). The solution should be stored at 4°C and should also be used within 4-6 weeks. For making 10 ml of antibody solution:

1. Dissolve 10 mg of bovine serum albumin (BSA, Fraction V) and 10 mg of IgG from rabbit serum (Sigma I5006) in 10 ml of 0.1 M phosphate buffer (3.3.2.2.).
2. Remove a 0.5-1 ml aliquot of this solution to later be used with the non-specific binding (background) tubes in the assay.
3. Add an appropriate volume of the JH antibody to the remaining RIA serum solution.

Note: The appropriate concentration of ecdysone antibody will have to be established in prior tests evaluating sensitivity through a dilution series of the respective antibody, remembering that an RIA is an isotope dilution assay whereby the specific antibody is the limiting factor. Ideal antibody concentrations should give maximal-binding cpm values corresponding to 35-40% of the total radioactivity added to each sample (around 1800-2200 cpm under the above described conditions). Such tests also need to be run periodically to ascertain binding and, if necessary, adjust the antibody concentration.

3.4.3. Running an ecdysteroid RIA

1. To set up a standard curve (in duplicates), prepare two series of 10 disposable borosilicate glass culture tubes (6 x 50 mm); to eight tubes each, add serial aliquots of the stock solution of unlabelled 20E, this being 0.5, 1, 2, 7.5, 10, 15, 20, 40 µl (corresponding to 25, 50, 100, 375, 500, 750, 1000 and 2000 pg of 20E).

Note that pipetting of the standard curve samples must be done with Hamilton-type syringes that are exclusively dedicated to use in this ecdysteroid RIA.

2. The remaining two tubes in each series do not receive any unlabelled JH; one will receive antibody-free background solution (to assess non-specific binding) and one will receive radiolabelled JH only (maximum binding values).

All further steps (3-27) are the same as those described for the JH RIA in section 3.3.3.

3.4.4. Data analysis

For data analysis and calculation of intra and inter-assay variation proceed as described for the JH RIA (see section 3.3.4.).

3.4.5. User Safety

The use of radioactive compounds ([23,24-3H(N)]ecdysone) requires authorization for individual user and laboratory according to national regulations and guidelines. Working with gloves is required to avoid direct contact with skin, but no further protection from radiation is necessary due to the low energy of tritiated compounds. Disposable material (glass) used during handling of radioactive compounds and liquid residues, as well as scintillation cocktail containing radioactivity must be properly disposed of following national guidelines. Dichloromethane and methanol are solvents harmful to health if used improperly. UV-light (254 nm) causes DNA damage and is especially harmful to the eyes; use protective glasses or a UV shield.

3.5. Measuring the rate of juvenile hormone biosynthesis by the paired *corpora allata*

This protocol describes a rapid partition radiochemical assays for quantifying rates of juvenile hormone III biosynthesis *in vitro* from honey bee *corpora allata* (CA), the glands responsible for JH production (Goodman and Cusson, 2012). Excised glands are incubated with methionine, which is a methyl donor to the ester function of the JH molecule (Metzler *et al.*, 1972; Judy *et al.*, 1973). Using radiolabelled methionine results in the production of radiolabelled JH III, which can then be quantified after partitioning into an isooctane phase. This technique was initially developed by Pratt and Tobe (1974) and Tobe and Pratt (1974), modified by Feyereisen and Tobe (1981) and adapted for use in honey bees (Rachinsky and Hartfelder, 1990, 1998; Huang *et al.*, 1991). This approach is most useful when quantification of the activity of the glands, rather than circulating JH levels, is desired. Its difficulties lie primarily in the ability to surgically remove and handle the glands without damaging them prior to incubation.

3.5.1. Purifying and preparing radioactive methionine for use in assay

Just prior to use, the labelled methionine will have to be washed to remove any unbound tritium, a source of contaminating "noise".

1. Dilute 100 µCi of L-[methyl-3H]-methionine (>97%, 1 mCi/ml, 70-85 Ci/mmol; PerkinElmer NET061X250UC) in insect saline to a final volume of 100 µl in a 1.5-ml microcentrifuge tube.
2. Add 500 µl of isooctane (2,2,4-trimethylpentane, anhydrous, 99.8%; Sigma-Aldrich 360066) to the methionine.
3. Mix well by vortexing for 1 min.
4. Centrifuge at 10,000 x g for 10 min.
5. Using a pipettor, remove the isooctane (i.e. upper) layer, avoiding to touch the lower methionine/saline layer.
6. Repeat the wash steps 2-5 two more times.
7. After the third wash, transfer 100 µl of isooctane to a scintillation vial to determine how much contaminate remains.
    7.1. Evaporate the isooctane in the vial to near-dryness under nitrogen.
    7.2. Add 3 ml of scintillation fluid with a high efficiency for tritium (e.g. ScintiSafe Econo F, Fisher Scientific) to the vial.
    7.3. Vortex for 1 min.
    7.4. Determine the radioactivity of the isooctane in a scintillation counter.
    7.5. If the background count is sufficiently low (i.e., <1000 dpm), the methionine is ready for use.
    7.6. If the background count is >1000 dpm, repeat the three wash cycle (steps 2-5).
    7.7. If the cleaning process does not reduce the background dpm under 2,000 within two wash cycles, incubate the methionine in 500 µl of isooctane overnight (or longer) to remove more of the degraded isotope.
8. Evaporate any of the isooctane remaining in the vial with nitrogen gas.
9. For ten samples and a negative control, dilute 65 µl of the methionine solution in 1235 µl of Grace's insect medium (from commercial supplier, e.g. Sigma-Aldrich) that is free of L-methionine.

This should provide a final (recommended) concentration of 100 µM methionine per incubation, with a specific activity of 5 µCi/100 µl.

Higher concentrations will ensure that radioactive methionine is incorporated into any JH synthesized, but will also increase the background counts.

3.5.2. Measuring the rate of JH biosynthesis

1. Remove the paired corpora allata (CA) from the base of the bee brain (see the section on isolation of the retrocerebral complex in the *BEEBOOK* paper on anatomy and dissection (Carreck *et al.*, 2013). While minimizing the amount of brain tissue attached to the glands, leave any attached tracheal elements as these enhance buoyancy. This will help ensure the glands stay near the surface of the medium during incubation, enhancing oxygenation and tissue activity (Kaatz *et al.*, 1985; Holbrook *et al.*, 1997).
2. Separately preincubate each dissected pair of CA in 100 µl of nonradiolabelled Grace's medium for 15 min in borosilicate glass culture tubes (6 x 50 mm) at room temperature.
3. Aliquot 100 µl of radiolabelled medium into a duplicate set of labelled glass culture tubes.
4. Using a small copper wire hoop, pick up a droplet of medium containing the preincubated CA and transfer it to the corresponding tube containing the radiolabelled medium. Ensure that the tissue stays close to the surface to enhance oxygenation.
5. Prepare a negative control tube containing radiolabelled media with no added tissue.
6. Loosely cover the tubes with plastic wrap to prevent desiccation, but ensure an adequate supply of oxygen.

7. Incubate the samples on a variable-plane mixer set at a 15°-17° angle at 90 rpm at 27°C for 3h.

The functional capacity of the *A. mellifera* CA begins to degrade within a few hours after dissection, so the 3h incubation allows for an accurate assessment of the rate of release and maximizes the amount of JH available for detection purposes.

8. At the end of the incubation period, remove the CA.
9. Add 250 µl of chilled isooctane to each tube.
10. Incubate on the variable-plane mixer for an additional 15 min.
11. Vortex the samples for 1 min.
12. Centrifuge at 10,000 x g for 10 min.
13. Using a pipettor with a gel loading tip, transfer 100 µl of the upper isooctane layer to each of two labelled scintillation vials so that each extract is assayed in duplicate.
14. Reduce the volume of the isooctane to near-dryness under nitrogen or in a vacuum concentrator.
15. Add scintillation fluid (see section 3.5.1., step 7.2) to the vial.
16. Vortex for 1 min.
17. Let rest for 1 h before measuring the radioactivity in a scintillation counter.

3.5.3. Data analysis

1. Subtract the average background count (from the negative control samples) from each reading to correct for background noise.
2. Determine the quantity of JH the CA released into the medium by converting the cpm value obtained from the previous step into dpm counts taking into account the tritium sensitivity of your scintillation counter (counter sensitivity is determined by running a manufacturer supplied scintillation vial containing a specified amount of the isotope of interest; correct for half-life).
3. Convert this dpm value into pmol JH released, taking into account the specific activity (typically 70 Ci/mmol) of your labelled methionine: considering that 2.22 dpm = 1 pCi, a dpm value of 155,400 = 1 pmol JH.
4. Calculate the hourly release rate by dividing the pmol value obtained (step 3) by the duration of the incubation period (divide value by 3 if incubation was 3 h).

3.5.4. User Safety

The use of radioactive compounds (L-[methyl-3H]-methionine) requires authorization for individual user and laboratory according to national regulations and guidelines. Working with gloves is required to avoid direct contact with skin, but no further protection from radiation is necessary due to the low energy of tritiated compounds. Disposable material (glass) used during handling of radioactive compounds and liquid residues, as well as scintillation cocktail containing radioactivity must be properly disposed of, following national guidelines. Isooctane is a flammable solvent.

4. Biogenic amine extraction and quantification by HPLC-ECD

4.1. Introduction

Biogenic amines have a variety of roles in the lives of insects, acting as neurohormones, neuromodulators, and neurotransmitters that can influence both behaviour and physiology (reviewed in Blenau *et al.*, 2001; Scheiner *et al.*, 2006). Four biogenic amines, dopamine (DA), octopamine (OA), serotonin (5-hydroxytryptamine, or 5-HT), and tyramine (TA), have been identified as being significantly associated honey bee behaviour and may be responsible for orchestrating the complex division of labour within their colonies (Fuchs *et al.*, 1989; Brandes *et al.*, 1990; Harris and Woodring, 1992, 1995; Taylor *et al.*, 1992; Bozic and Woodring, 1998; Wagener-Hulme *et al.*, 1999; Scheiner *et al.*, 2002, 2006; Schulz *et al.*, 2002; Barron and Robinson, 2005; Fussnecker *et al.*, 2006; Agarwal *et al.*, 2011). Several approaches have been used to measure amine concentration in insect central nervous system tissues (histofluorimetry, immunohistochemistry, radioenzymatics, gas chromatography-mass spectroscopy), but one that has been used most frequently in honey bees is high performance liquid chromatography coupled to electrochemical detection (HPLC-ECD). This approach utilizes a carrier solution, referred to as the mobile phase, to bring the sample across a filter column and then to the detector. HPLC-ECD analysis can be very sensitive and provide robust and repeatable results. The primary issue with this approach is that HPLC systems can be difficult to maintain over time, necessitating the processing of samples in small batches and running calibrating standards frequently.

An important caution with any approach used to quantify biogenic amines is that their levels can change rapidly in bees subjected to any of a variety of environmental perturbations (Harris and Woodring, 1992; Harris *et al.*, 1996; Chen *et al.*, 2008). For that reason the handling time when collecting samples should be as short as possible, and then technique used should be very uniform. In addition, these amines are highly sensitive to light and heat, therefore every precaution should be made to keep samples cold and covered prior to analysis.

4.2. Dissection of the brain from the head capsule

While it is sometimes possible to take measurements from a single individual, the most robust and reliable results come from pooling multiple individuals. Depending on the size of the brain sections being used, tissue from up to 20 individuals may be needed per sample. Because the stress of being handled can affect neurotransmitter expression, collect the bees as quickly as possible. Frozen bees can be dissected immediately (preferable) or temporarily stored in liquid nitrogen. See the section on dissection of the brain from the head capsule in the *BEEBOOK* paper on anatomy and dissection (Carreck *et al.*, 2013) for the method to extract the brain out of the head capsule.

Place the desired brains or brain sections into a labelled 0.5 ml microcentrifuge tube. Cover and store at -80°C for up to several weeks if the samples are not to be analysed immediately.

4.3. Pre-analysis preparation of the HPLC

1. Prepare the mobile phase (1l) as follow:
    1.1. 700 ml polished water (this is ddH_20 from which all impurities were removed)
    1.2. 150 ml methanol
    1.3. 150 ml acetonitrile
    1.4. 0.433 g sodium dodecyl sulphate (SDS)
    1.5. 10.349 g sodium phosphate monobasic
    1.6. 1.47g sodium citrate
    1.7. Adjust pH to 5.6 using phosphoric acid, delivered a drop at a time while stirring.
2. Degas the mobile phase for the HPLC in an ultrasonic bath for 5 min prior to running any samples to prevent air from entering the column and detector cells.
3. Set the mobile phase flow rate to 1.0 ml/min (normal pressure ~95 bar; set max pressure to 140 bar).
4. Apply current to the ECD cells (e.g. Channel 1, 650mV; Channel 2, 425mV; Channel 3, 175mV; Channel 4, -175V).

The amount of current per channel will need to be adjusted to optimize peak responsiveness as per the manufacturers recommendations; it will take approximately 45 min for channel voltage to equilibrate.

5. During the equilibration process, prepare the external (ES) and internal standards (IS).

4.4. Preparation of internal and external standards

The internal standard for DA and 5-HT is 3,4-dihydroxybenzylamine (DHBA), while that for OA and TA is synephrine. Because of the instability of amines in solution, new standards should be made up daily to ensure accuracy. However, the stock dilutions may be relatively stable for a few days of use if they are kept properly chilled and shielded from light.

4.4.1 Stock dilutions (1 x 10^6 pg/µl)

For each amine:

1. Weigh out the specified amount (these values are adjusted for the weight of associated HCl and HBr molecules) into separate, labelled 12 ml glass vials.
2. Dissolve the standards in:
    2.1. 9.975 ml chilled 0.2M perchloric acid (PCA; 29.6 ml 70% perchloric acid in 1l polished water) and
    2.2. 25µl 0.1M EDTA solution (3.722 g EDTA in 100 ml polished water)
3. Cover the vials in foil to exclude light.
4. Refrigerate (4°C).

4.4.2. External and Internal standard

External Standards (ES)	Internal Standards (IS)
DA: 0.0124 g	DHBA: 0.0174 g
OA: 0.0124 g	Synephrine: 0.0100 g
5-HT: 0.0121 g	
TA: 0.0127 g	

4.4.2.1. Dilution ES-A

to 7.775 ml chilled 0.2 M PCA add:

- 2.0 ml DA (2000000 pg/µl),
- 25 µl OA (2500 pg/µl),
- 25 µl 5HT (2500 pg/µl),
- 125 µl TA (12500 pg/µl)

4.4.2.2. Dilution IS-A (5000 pg/µl):

to 9.925 ml chilled 0.2 M PCA add

- 50 µl DHBA (50000 pg/µl),
- 25 µl Synephrine (25000 pg/µl)

4.4.2.3. Dilution IS-B (500/250 pg/µl)

add 10 µl of IS-A to 990 µl chilled 0.2 M PCA; this will be used for extracting brain amines.

4.4.2.4. Standard Curve:

- Dilution B: Add 10 µl IS-**A** and 15 µl ES-**A** to 945 µl PCA. (3000 pg/µl)
- Dilution C: Add 10 µl IS-**A** and 12 µl ES-**A** to 954 µl PCA. (2400 pg/µl)
- Dilution D: Add 10 µl IS-**A** and 9 µl ES-**A** to 963 µl PCA. (1800 pg/µl)
- Dilution E: Add 10 µl IS-**A** and 6 µl ES-**A** to 972 µl PCA. (1200 pg/µl)
- Dilution F: Add 10 µl IS-**A** and 3 µl ES-**A** to 981 µl PCA. (600 pg/µl)
- Dilution G: Add 10 µl IS-**A** and 1 µl ES-**A** to 987 µl PCA. (200 pg/µl)
- Dilution H: Add 10µl IS-**A** to 990µl PCA.

4.5. HPLC separation of standards

Once the ECD cells equilibrate, standard samples can be run on the HPLC system.

1. Prior to injecting any samples, prime the HPLC by injecting 10 µl of 0.2 M perchloric acid (PCA).
2. After this and every injection, be sure to flush the syringe with polished water several times.

Any residual PCA would crystallize, causing the plunger to become frozen in the barrel. As an added precaution, store the plunger out of the syringe when not in use.

3. If the PCA run looks clear of peaks, sample analysis can begin.

Standards and samples should be processed in this order: Standards G to B (lower to higher concentration), Standard H, 5-7 samples, Standards G/D/H, 5-7 samples, Standards G/D/H, PCA Blank. Standard H and the PCA clear the HPLC of residual amine peaks. Standards should always be run at the start, middle and end of any series to ensure that the sensitivity of the system has not changed. The cycle can be repeated as necessary. Duration of the run will depend on the flow rate, which in turn is determined by the pressure registered within the HPLC. Adjustments to flow rate and run length may need to occur over the course of the day as contaminates injected with the sample cause a gradual pressure increase.

Because of the sensitive nature of the amines, an autoloader cannot be used to inject the samples, as they would degrade while waiting. Instead, use a microsyringe to directly deliver the sample into the injector port.

While standards are being run on the HPLC, tissue samples can be prepared for analysis.

4.6. Sample preparation

1. Remove the stored brains from the freezer or liquid nitrogen and place them in a covered ice bucket.
2. Add 20 µl of chilled IS-B solution to each tube.
3. Homogenize each brain using a separate tissue grinder.
4. Remove the tissue and liquid from the pestle to ensure that all tissue remains in the solution.
5. Suspend the sample tubes in an ultrasonic bath filled with ice water.
6. Cover to reduce light exposure.
7. Sonicate the tubes for 5 min to disrupt tissue.
8. Leave the tubes covered and in ice water for an additional 20 min to allow the amines to be extracted from the brain tissue.
9. Centrifuge the tubes at 12,000 RCF for 10 min at 4°C.
10. Collect the supernatant and pass it through a 0.22-µm nylon membrane filter into a fresh, labelled microcentrifuge tube. This step will help remove any remaining tissue, protecting the HPLC system from clogging.
11. Store the tube containing the supernatant on ice and protected it from light until used.
12. Place the original tube containing the residual brain tissue in a -80°C freezer for later quantification.

4.7. Separation and quantification of biogenic amines by HPLC

For each standard and sample:

1. Load 10 µl of the collected supernatant into the HPLC system.
2. Identify the amines in an HPLC trace by determining the retention time and relative peak sizes for each of the detector channels.

The exact retention times for the amines will vary according to analysis conditions, but they do occur in the specific order given in the example below.

3. For each amine, determine the area of the peak for the one channel on which it was largest.

An example of amine characteristics follows. Although the order in which these peaks appear will remain constant, the specific times and channel responses will vary depending on the HPLC system, flow rate, and applied currents:

- Octopamine (OA): 5.2 min, big Channel 1 peak, smaller Channel 2 peak
- DHBA: 5.5 min, big Channel 2 peak, smaller Channels 1 and 3 peaks
- Synephrine: 6.0 min, big Channel 1 peak, smaller Channel 2 peak
- Dopamine (DA): 6.6 min, big Channel 2 peak, smaller Channel 1 and 3 peaks
- Tyramine (TA): 9.6 min, big Channel 1 peak, smaller Channel 2 peak
- Serotonin (5-HT): 10.2 min, big Channel 2 peak, smaller Channels 1 and 3 peaks

4. If the standards maintained comparable values through multiple batches of samples, a single standard curve can be calculated from their combined data. If the standard values changed over time, each batch of samples will need to be compared to a curve calculated from the standards run just prior to and immediately following each batch.

In order to be sure that any differences in amine content are due to true physiological differences and not just differences in the quantity of tissue from which the amines were extracted, it is necessary to standardize the amine content against tissue content. Several approaches are available to determine protein content, such as the Bradford or Lowry assays (see section 1.2), but the accuracy of many of these assays is compromised by the minute volume of tissue involved in an individual brain. An alternative and simple approach is to determine the dry mass of the brain tissue.

To quantify brain mass:

1. Remove the residual tissue pellet from the sample tube.
2. Place it on a pre-weighed and labelled 1cm square section of foil.
3. Fold these squares over to secure the sample.
4. Place them in an oven set to 100°C.
5. Bake the samples for 24 h.
6. Weigh the squares on a scale that can measure microgram differences.

5. Temperature, radiation and humidity measurement in honey bees

Temperature is a decisive parameter in honey bee development, physiology and behaviour, at the individual as well as at the social level (Heinrich, 1993). Larval respiration and growth is strongly dependent on temperature (Petz *et al.*, 2004). A high speed of

development is guaranteed via social regulation of brood temperature in the range of 33-36°C (Kleinhenz *et al.*, 2003; Stabentheiner *et al.*, 2010). Low temperatures during pupal development may impair learning and cause significant differences in the behaviour of adult bees (Tautz *et al.*, 2003; Becher *et al.*, 2009). Colony temperature also affects the basal metabolism of hive bees (Stabentheiner *et al.*, 2003a; Kovac *et al.*, 2007). Social thermoregulation allows for overwintering of colonies in cold climates (Stabentheiner *et al.*, 2003b). During the foraging cycle honey bees are always endothermic, which means that they display a high oxygen consumption (and thus energy turnover) to keep their body temperature well above the ambient level (Heinrich, 1993; Stabentheiner *et al.*, 1995; Kovac and Schmaranzer, 1996; Roberts and Harrison, 1999; Kovac *et al.*, 2010; Stabentheiner *et al.*, 2012). Measurement of honey bee body and colony temperature and of environmental parameters requires care to achieve correct results. The following summary is meant as a guide to avoid the main pitfalls of honey bee body temperature and environmental parameter measurement.

5.1. Contact thermosensors

There is a great variety of contact thermosensors available. For the measurement of ambient temperature, sensor size is not so critical, though smaller sensors react much faster to changes in ambient temperature than larger ones. For honey bee body temperature measurement, however, it is *indispensable* that they are not just small but tiny! Otherwise, large measurement errors are to be expected. Furthermore, for measurement of brood nest (comb) temperature, care has to be taken to place the thermosensors in the correct (desired) position, e.g. in the actual centre of the brood comb.

5.1.1. Thermocouples and thermoneedles

Thermocouples are standard sensors for the measurement of ambient temperature. Their small (i.e. thin) size and flexibility allows installation of many sensors in a honey bee colony (Stabentheiner *et al.*, 2010). Due to their usually small sensor tip – and thus heat capacity – their reaction to temperature changes is fast. Sensitivity and reaction speed can be maximized by using small wire diameters (ones close to 12 µm are available; for example see Omega Engineering Inc., http://www.omega.com). Accuracy depends primarily on the electronic thermometer in use, including the accuracy of the reference (cold) junction compensation in the connector (junction) block. During outdoor measurements in sunshine, the electronic thermometer may not be exposed to solar radiation. Thermal gradients in the connector (junction) block may lead to high measurement errors (up to several °C). Similar errors may occur if ambient temperature changes rapidly, for example when stepping outside. Equilibration of the device is always recommended for utmost accuracy.

5.1.1.1. Thermocouple types

There are different types of thermocouples, with different combinations of wire metals, e.g. NiCr/NiAl (Type K), Cu/Constantan (Type T), etc. Thermocouple types may differ considerably in sensitivity (and, irrelevant for biological applications, measurement range). Make sure to always use the correct type of thermocouple, matching the type and size of connector input on your electronic thermometer.

For honey bee body temperature measurement, thermocouple wires have to be thin (diameter <70 µm). For "grab and stab" measurement of body (core) temperature (Stone and Willmer, 1989) the thermocouples have to be inserted within hypodermic needles. Most practical are thermocouples inserted in "thermoneedles" by the manufacturer (available ready for use and in different sizes for example at Omega Engeneering, Inc.).

5.1.1.2. Calibration

It is an intrinsic property of thermocouples that their characteristic curve is neither linear nor does it follow a persistent function throughout the measurement range (Fig. 5). Modern thermocouple thermometers, however, usually apply appropriate corrections automatically. If an accuracy of better than 0.3°C has to be guaranteed, individual calibration of each thermocouple is necessary (e.g. in a water bath against an accurate laboratory thermometer). In the temperature range relevant in honey bee life, of about -30 to 100°C, linear correction functions are sufficient. Advanced data loggers provide the possibility to store the corrections permanently in a device or connector memory (e.g. Ahlborn Messtechnik, http://www.ahlborn.com).

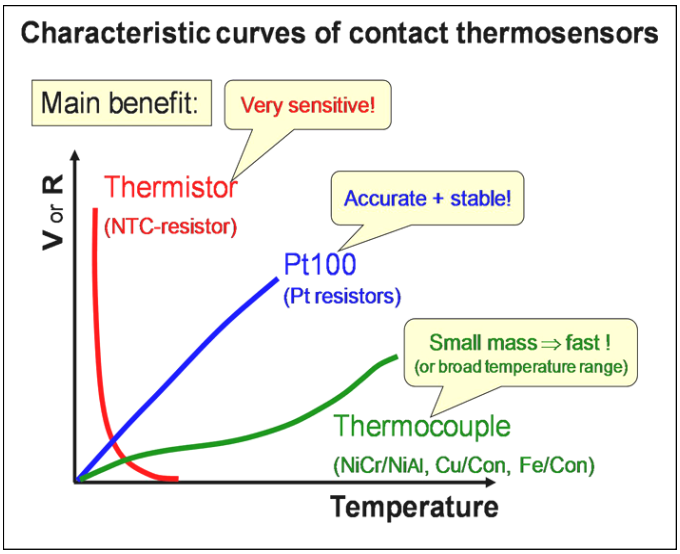

Fig. 5. Characteristic curves of the main types of contact thermosensors (schematic), with main benefits. For main constraints see text. Measured parameters: Voltage (V) or resistance (R, in Ohm).

Graph by Anton Stabentheiner

For air temperature measurements in direct sunlight, heating of the thermocouple junction by solar radiation has to be taken into consideration. To avoid any resulting measurement error, a calibration comparing the readout between two thermocouples, one in sunshine and one in the shade, has to be applied (Stabentheiner *et al.*, 2012). To do this properly, the intensity of solar radiation also has to be measured (see section 5.4.).

5.1.1.3. Use inside colonies

When using probes within colonies, these may require protection from direct contact with the bees, so as to avoid heating by direct bee contact (compare Kleinhenz *et al.*, 2003; Stabentheiner *et al.*, 2010). Logging temperature data at short time intervals helps to uncover such events which will appear as temperature peaks. The actual sensory spot of a thermocouple is the junction between the two wire metals that is closest to the electronic thermometer. This is usually the thermocouple tip. However, hive bees often gnaw at the wire insulation. This is not critical as long as the wires do not touch. But should they touch, location of temperature measurement may change.

5.1.2. Thermoresistors (thermistors, Pt100)

Thermistors and Pt100 thermosensors are not sensitive to thermal gradients within the electronic thermometer. Their size, however, is usually larger. The advantage of thermistors is their high sensitivity (Fig. 5). The strong nonlinearity of their characteristic curve is considered properly by modern electronic thermometers. The strong nonlinearity of the characteristic curve of thermistors, however, implies that their sensitivity decreases with increasing temperature. Arrays of many thermistors may be used to monitor the temperature of many comb cells simultaneously (Becher and Moritz, 2009). If a high accuracy and long-term stability is required, Pt100 sensors (platinum resistors with 100 Ohms at 0°C) are a good choice. In any case, individual calibration is required for utmost accuracy.

5.2. Non-contact temperature measurement

5.2.1. Infrared spot thermometers

Infrared spot thermometers may be used for *surface* temperature measurement of larger targets, such as combs, hive walls, soil, etc. The size of the measurement spot increases with distance to the measured object. In most instruments, spot size is too large for body temperature measurements in honey bees. Some devices offer a close -up lens for spot size reduction. Spot size, however, has to be *considerably* lower than the honey bee thorax diameter of ~4 mm, because accurate measurements in spherical objects are only possible at an angle smaller than ~30° from the normal to the surface. Care has to be taken that a built-in laser indicator for measurement spot identification really hits the target in close vicinity to the measured object. Parallax errors may lead to false measurements. Nowadays, however, handheld thermographic cameras (see section 5.2.2.1.) have become so cheap that there is no need of using such devices for honey bee body temperature measurement. In fact, they are mostly useless for this purpose.

5.2.2. Infrared thermography

In insect thermoregulation research, infrared (IR) thermography provides several advantages over conventional thermometry. Most importantly, the bees do not have to be killed, as required in measurements with 'thermoneedles'. They neither need to be touched, nor impaired in their natural behaviour. Measurements on the same individual can be repeated several times, and the spatial distribution of the body surface temperature can be evaluated simultaneously (Stabentheiner *et al.*, 1987, 2012). A 'disadvantage' is that only surface temperatures, but not internal body temperatures are obtained.

5.2.2.1. Main thermographic camera types

In earlier times, the infrared (IR) radiation of an object to be measured was scanned by rotating prisms or mirrors and focussed onto a single detector (detector material for example InSb or HgCdTe). Nowadays, many detectors are arranged in focal plane arrays (FPAs). Data readout ('scanning') from the chip is done electronically. Spatial resolution of FPAs may be of 120 x 120, 320 x 240 (see Stabentheiner *et al.*, 2012) or 640 x 480 pixels, or even reach 1280 x 1024 pixels in (rather expensive) top camera models.

There are two main types of thermographic cameras for the practical use in honey bee body temperature measurement. Cameras either use photon-counting detectors, like InSb, PtSi, HgCdTe, or QWIP (quantum well interference photodetectors), or microbolometer arrays. Cameras with photon-counting detectors measure infrared photons directly. To achieve their (usually) high sensitivity (<20 mK in some InSb cameras for example) they require detector cooling. While in earlier times detectors were cooled with liquid N_2 or expanding Ar gas, cooling is nowadays done with miniaturized built-in compressors. InSb and QWIP chip cameras are usually fast in reaction (have a short exposure time), which make them especially convenient for the measurement of flying (or fast moving) honey bees.

Cameras with microbolometer arrays measure infrared radiation indirectly, via (small) temperature changes of the detector pixels. Detector materials in use are for example Vanadium oxide (VOX) or amorphous Silicon (aSi). A disadvantage of (earlier) bolometer cameras is that they may exhibit considerable drift due to internal temperature changes (Stabentheiner *et al.*, 2012). This may require frequent internal and external recalibration. Modern cameras often have a better (though not complete) drift compensation. By now, the sensitivity of the top bolometer cameras is better than 0.03 K. Furthermore, small and relatively cheap handheld bolometer cameras have become available. Many of these allow to set the focus close

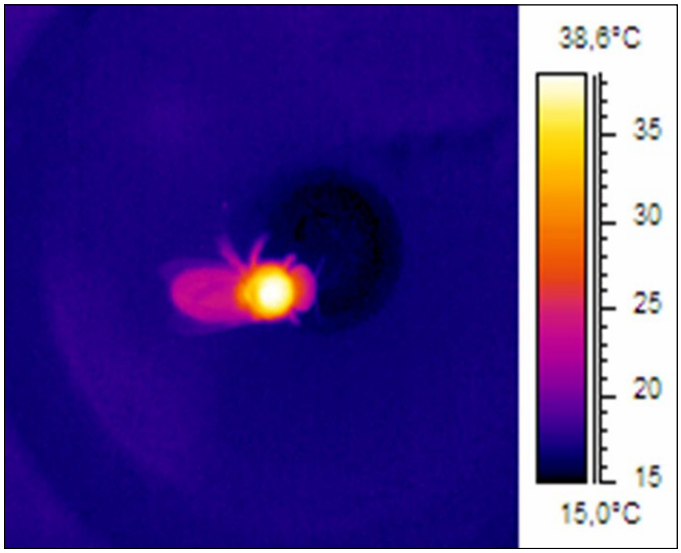

Fig. 6. Thermogram of a honey bee sucking 0.5 M sucrose solution from an artificial flower. Body surface temperatures: head = 26.1°C, thorax = 38.6°C, abdomen = 23.3°C. Ambient air temperature close to the bee: 16.2°C, ambient radiation: 18.1 W m^{-2}, ambient humidity: 40.6%. Thermographic camera: FLIR i60 (180x180 microbolometer detector). For instrument accuracy see Stabentheiner *et al*. (2012).
Image by Anton Stabentheiner

enough to measure surface temperatures of thorax, abdomen, and head of honey bees (Fig. 6).

The main spectral ranges of thermography cameras in use are the 3-6 µm (short wave) and the 7-14 µm (long wave) wavebands. Short-wave cameras (with e.g. InSb or PtSi FPA) are fine for laboratory use. For field measurements, long wave cameras are recommended (e.g. microbolometer or QWIP FPA), as these are much less sensitive to reflected solar radiation (see next section).

5.2.2.2. Honey bee cuticle infrared emissivity

Thermographic measurement of the surface temperature of an object requires knowledge of its infrared surface emissivity. This quantity is a quotient (ranging from 0-1) describing how much radiation an object emits at a certain temperature in comparison to an ideal black body radiator, which emits the theoretical maximum. The emission and absorption of infrared radiation are directly coupled according to Kirchhoff's law, which means that an opaque surface with an emissivity of 0.97 also has an absorptance of 0.97 (i.e. 97% of incoming infrared radiation is absorbed and 3% are reflected). Therefore, the emissivity value can be used to compensate for ambient infrared radiation reflected into the camera via the object, which adds to the radiation emitted by the object and therefore would produce a measurement error if left uncorrected.

The honey bee cuticle has an infrared emissivity of ~0.97 (Stabentheiner and Schmaranzer, 1987), so approximately 3% of the ambient infrared radiation is reflected via the cuticle into the infrared camera. With standard evaluation software provided with thermography cameras, this can be compensated by considering ambient 'black body' temperature, and this is usually done by measuring ambient temperature.

During measurements in sunshine, however, there is an additional error caused by solar radiation reflected by the cuticle, which is not corrected for by the above mentioned correction of reflected ambient infrared radiation. This effect can be measured by thermographing insects at different intensities of solar radiation with a real-time infrared camera (frame rate > 30 Hz). The cuticular surface temperature is first measured in sunshine and then the insect is shaded and its temperature measured again within a few milliseconds. The resulting measurement error turns out to be small in the 7-14 µm waveband (long wave cameras). The applied correction factor for reflected solar radiation amounted to 0.2183°C kW^{-2} m^{-2} (Stabentheiner *et al*., 2012). However, with short wave cameras (3–6 µm waveband, which is closer to the solar radiation maximum in the visible range) the measurement error may be considerably higher (several degrees). Therefore, long-wave cameras should be used for field measurements in sunshine.

5.2.2.3. Thermography camera calibration with reference radiator

Even rather cheap microbolometer cameras have a sensitivity of <0.1°C. This means that they can sense temperature differences between different areas within a picture with this thermal resolution. Measurement accuracy of most cameras, however, is only 2°C or 2% (whatever is smaller). This is true for cameras with both photon counting and microbolometer detector chips. In special cases, for example if macro lenses are used, these specifications may not be valid some time after the camera was turned on or at certain ambient conditions (Stabentheiner *et al*., 2012). Only a few models offer an accuracy down to 1°C. It has to be kept in mind that this refers to the internal instrument accuracy. Errors resulting from wrong input of surface emissivity (see section 5.2.2.2.) or environmental data (ambient temperature, relative humidity, distance to the measured object) may add to these values. Therefore, calibration with an external reference radiator is indispensable if an accuracy of better than 2°C has to be guaranteed.

A simple precision cavity black body reference radiator of high accuracy (<0.2°C) can be constructed by immersing a hollow metal cylinder (e.g. brass) in a regulated laboratory water bath (Stabentheiner and Schmaranzer, 1987) or, for field use, in a thermal coffee pot filled with warm water (Fig. 7). Its inner surface has to be covered by matt 'black' lacquer (infrared emissivity should be at least 0.95; e.g. Nextel Velvet Coating 811-21). The cavity increases the apparent cavity emissivity to a value >0.995, which is close to an ideal black body radiator (emissivity = 1), if the ratio of cylinder depth to radius is at least 10:1 (Sparrow and Cess, 1970). The accuracy of such a black body depends primarily on the accuracy of the water

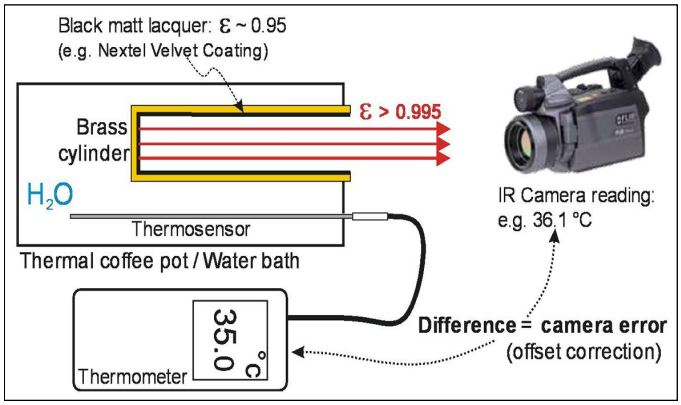

Fig. 7. Infrared camera offset calibration with a cavity black body radiator. Dimensions of the brass cylinder: ratio of cylinder depth to radius ≥ 10:1. ε = infrared emissivity (see section 5.2.2.2.).

Graph by Anton Stabentheiner

bath temperature measurement. An electronic thermometer or a laboratory thermometer with 0.1°C scaling should provide the desired accuracy. Reference temperature should be at least 5°C above ambient temperature for utmost accuracy.

Since thermography cameras may display a drift over time, camera calibration should be performed as often as possible. Correction works best if the reference radiator is permanently visible in the infrared pictures (Stabentheiner *et al.*, 2012).

5.2.2.4. Attenuation of infrared transmissive films

If honey bee body temperature has to be measured inside a hive it is often necessary to do this through infrared transmissive films to prevent the bees from leaving the colony (Stabentheiner *et al.*, 2003a, 2010). Good choices are polypropylene or cellophane films used by florists for wrapping of flowers. They provide good stability and transparency. Polyethylene would be even more transparent to infrared radiation, especially in the 'long-wave' (7–14 µm) spectral band. However, when testing commercially available films, these were mostly of rather poor 'optical' quality, i.e. their surface was not sufficiently smooth. The attenuation of the infrared radiation by the transmissive film can be compensated for by covering part of the reference source with a stripe of the same film (Stabentheiner *et al.*, 2012; and section 6 of this paper). By doing so, camera calibration with the reference radiator compensates for the attenuation of the film, and errors resulting from ambient reflections *via* the film surface can be minimized.

The attenuation of infrared transmissive films may also, with less precision, be corrected by determination of a 'special atmospheric transmission' coefficient (which usually compensates for infrared absorption of the atmosphere at greater measurement distances). This can be done by comparing the direct radiation of a black body radiator (Fig. 7) with radiation passing through the film. An iterative change of the atmospheric transmission coefficient for the film measurement, in the camera or in the evaluation software, until the temperature reading equals the direct measurement provides the correction coefficient.

Special care has to be taken to avoid different reflection of ambient radiation by room walls, IR camera, operators, etc. from different parts of the film surface. In outdoor measurements, reflection of the sky via the film may produce considerable measurement errors. For more details see Stabentheiner *et al.* (2012). In general, however, it is worthy of note that thermographic measurement through plastic films should be avoided whenever possible because it inevitably adds additional sources of error.

5.3. Operative temperature

In field investigations of honey bee thermoregulation, operative temperature (T_e) provides an integrated measure of the action of several environmental factors on honey bee thermoregulation (Fig. 8). T_e is the body temperature of dead honey bees exposed to the same environmental conditions as the living bees under investigation. It allows separating the endogenous part of thermoregulation (endothermy) from the effect of environmental parameters on body temperature (Kovac *et al.*, 2010). These environmental parameters are ambient air temperature, radiative loss and gain of heat, thermal convection and external air convection (wind), and evaporative heat loss, mainly through the cuticle. In such experiments, dead honey bees are used as thermosensors (Fig. 8). T_e has been determined by using both dried or fresh carcasses. However, using fresh carcasses is recommended, because the use of dried bees strongly reduces honey bee heat capacity (because of their smaller mass), and this way considerably changes the reaction of the T_e-thermometer to environmental factors. For a detailed discussion of this topic see Materials and Methods in Kovac *et al.* (2010).

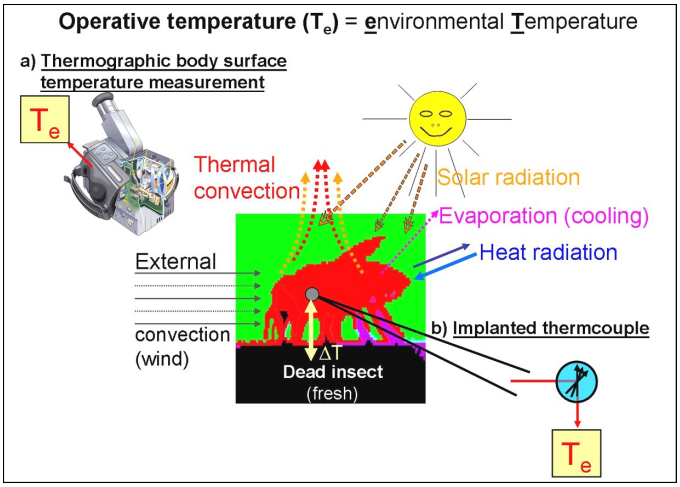

Fig. 8. Operative temperature (T_e) measurement in honey bees. T_e is the body temperature of dead bees exposed to the same environmental conditions as the living individuals under investigation. It can be measured either thermographically as surface temperature (a) or by an implanted thermocouple as core temperature (b).

Graph by Anton Stabentheiner

5.4. Radiation sensors

Ambient radiation acting on honey bees is sunlight in the visible and near infrared range (ca. 300-3000 nm wavelength, with the maximum in the visible range) and heat radiation in the middle to far infrared range (ca. 3-20 µm wavelength, with the maximum at about 8.5-11 µm at biologically relevant temperatures). The global radiation is the radiation from the upper hemisphere to a horizontal surface. It is the sum of the direct solar and the diffuse sky radiation (in W m^{-2}). Global radiation sensors measure in the solar spectrum from 0.3 to 3 µm wavelength.

5.4.1. Star pyranometers (according to Dirmhirn)

With star pyranometers, which are widely used in meteorology, the intensity of global radiation (radiant intensity) is measured indirectly, via measurement of the temperature difference between black and white copper plates arranged in the horizontal plane (star shape). The (solar) radiation heats the black plates stronger than the white ones. This temperature difference is measured using a thermopile (i.e. a serial arrangement of thermocouples) attached to the underside of the surfaces. This differential measurement principle minimizes the influence of ambient temperature. The advantage of star pyranometers is their robustness and a broad spectral range. Their main disadvantage is their rather inert reaction to radiation changes.

5.4.2. Photoelectric pyranometers

Photoelectric radiation probes measure the irradiance in the solar spectrum in the visible range and in the short-wave infrared range. Through filtering of the incoming radiation, their spectral range is tuned to the range of wavelengths of interest, e.g. UVB, UVA, visible light. Photoelectric global radiation sensors measure both direct and diffuse solar radiation. They usually cover the spectral range from 400 to 1100 nm. The spectral sensitivity curve should be flat for optimal results. Some companies manufacture miniaturized versions on request (see Stabentheiner *et al.*, 2012)

5.4.3. Measurement of the short-wave radiation balance

For energy balance measurements of foraging honey bees it may be necessary to determine the reflecting ability (albedo) of the ground surface in addition to the direct solar radiation. A combination of a pair of global radiation pyranometers, one directed upwards and the other downwards, allows for measurement of the short-wave radiation balance. The downward directed sensor measures the radiation reflected by the ground. The upward directed sensor measures direct and indirect solar radiation. From the readings of the two pyranometers, the albedo can be calculated.

5.5. Humidity measurement

For humidity measurements, capacitive humidity sensors are in common use. They are easy to use and of sufficient accuracy. They measure the effect of humidity on the dielectric constant of a polymer or metal oxide material. Advanced measurement devices provide calculation of absolute humidity (g m^{-3}), dew point temperature (°C), etc., in addition to relative humidity (%). For outdoor measurements, the probe has to be kept in the shade. The sun will heat the internal temperature sensor, which leads to false calculation of relative humidity.

For application within honey bee colonies miniaturized sensor heads (with a diameter of a few mm; see Kovac *et al.*, 2010; Stabentheiner *et al.*, 2012) are of great advantage. The use within colonies requires protection of the probe from the bees. Otherwise they may cover it over with wax and propolis.

6. Respiration and energetics measurement in honey bees

Respiratory gas exchange is one of the basic physiological mechanisms of insect metabolism. In honey bees, basal or resting metabolism and its temperature dependence (Stabentheiner *et al.*, 2003a; Petz *et al.*, 2004; Kovac *et al.*, 2007), discontinuous gas exchange cycles (Kovac *et al.*, 2007) and energetics of foraging and flying (e.g. Wolf *et al.*, 1989; Harrison and Hall, 1993; Harrison *et al.*, 1996; Woods *et al.*, 2005) have all been investigated so far. Respiratory measurements are also a valuable tool for the determination of (respiratory) critical thermal maxima and minima (CT_{max}, CT_{min}) and chill coma temperature (Lighton and Lovegrove, 1991; Lighton and Turner, 2004; Kovac *et al.*, 2007; Käfer *et al.*, 2012). Measurements on tiny honey bee larvae (Petz *et al.*, 2004) however require a different setup than measurements on flying (e.g. Roberts and Harrison, 1999) or thermoregulating bees (Goller and Esch, 1991; Blatt and Roces, 2001; Stabentheiner *et al.*, 2003a, 2012), bee groups (Moritz and Southwick, 1986; Southwick and Moritz, 1985, 1987), or whole bee swarms and colonies (Heinrich, 1981a, b; Southwick, 1985, 1988; Van Nerum and Buelens, 1997; see Heinrich, 1993 for more literature). Metabolic differences exist among stages of honey bee development. They are not just a result of differences in mass, but may reflect the bees' development of mitochondrial capacity and enzymatic make-up, as is the case in the first days after emergence (Hersch *et al.*, 1978; Harrison, 1986; Moritz, 1988). Honey bees are also subjected to environmental variation throughout their life cycle, and measurement of whole-body and whole-colony respiration and heat production can provide insights into respiratory and energetic adaptations to the challenges of environmental variation and contribute to a better understanding of the benefits of social cooperation.

This paper is meant as a short guide into the main concepts and setups of whole-animal and colony respiratory and energetic measurements in honey bees. It cannot, however, provide a complete and detailed description of all possible measuring arrangements. A

valuable help for a deeper understanding of the possibilities and limitations of insect respiratory and energetic measurement is given by Lighton (2008). For details on the measurement of cellular and sub-cellular respiration and metabolism good references are Suarez *et al.* (1996, 2000) and Suarez (2000).

Any company names mentioned herein are meant as a quick help for the reader to find a faster entry into the field. In no way it means that these companies are the only manufacturers of a certain type of device. Furthermore, since such information may change with time, the reader interested such equipment will eventually have to search for more and updated information.

6.1. Flow-through respirometry

Honey bees are, as honey bee scientists know quite well, extremely social insects. This means that their behaviour may change considerably if they are separated from the community to be put into a respiratory or energetic measurement chamber. In such situations individuals want nothing but find an exit out of the chamber to fly home. This may not matter (much) in experiments where flight energetics (Wolf *et al.*, 1989; Harrison *et al.*, 1996; Woods *et al.*, 2005) or the interrelation of thermoregulation and heat production are under investigation (Stabentheiner *et al.*, 2003a, 2012). Respiratory and energetic measurements also resemble a natural situation quite well if the bees have freely entered the measurement chamber because they expected a reward therein (Balderrama *et al.*, 1992; Moffatt, 2000; Stabentheiner *et al.*, 2012). It is *not* possible – and this has always to be kept in mind –, however, to determine the energy turnover of hive bees directly from respiratory measurements of isolated individuals. Energy turnover may change by more than a 100-fold due to an unpredictable degree of endothermy.

In research on insect respiration and energetics, flow-through respirometry is the state of the art (Lighton, 2008). Its great advantage is that the bees are supplied with fresh air throughout the experiment, which makes possible measurements over extended time periods (Kovac and Stabentheiner, 2007). If necessary, the atmospheric composition may be changed artificially, for example to investigate the impact of tracheal mites on flight capability (Harrison *et al.*, 2001) or of increased hive CO_2 levels on respiration and energy turnover. Modern equipment provides high sensitivity, down to the ppm (O_2 sensors) or sub-ppm range (CO_2 sensors). With appropriate calibration and care measurement accuracy of a few ppm is possible.

6.1.1. Measurement arrangements, measurement chambers and accessories

6.1.1.1. General setup

For respiration and energetics measurements the setup has to be adapted according to the questions under investigation, the developmental stage of the bees and the quantity to be measured. In open-flow investigations of insect respiration, different parallel and serial plumbing setups are in use. For general advice see also Lighton (2008) and Lighton and Halsey (2011). In most cases, scientists will be interested in O_2 consumption or CO_2 production of bees, mostly in comparison to ambient air, rather than in the measurement of absolute concentrations of these gases. Therefore, differential setups, comparing the gas concentration before and after a measurement chamber are the most relevant ones. A differential measurement setup which allows a fast, (semi-) automated switch between different measurement modes (serial or parallel; see sections 6.1.1.3. and 6.1.1.4.), and simultaneous thermographic measurement of body surface temperature is shown in Stabentheiner *et al.* (2012). However, setups with less automation will normally be sufficient to answer a specific question. In general it has to be noted that respiratory equipment is usually not of a 'buy and go' type. Every measurement situation requires its own adaptations. However, many companies provide solutions and help to fit the experimenter's requirements.

1. *Standard conditions:* The measurement output of instruments (volume of O_2 consumption or CO_2 production, or flow rate) usually refers to standard (STPS) conditions (0°C, 101.32 kPa = 760 Torr).
2. *Calculation of O_2 consumption (VO2) or CO_2 production(VCO2):* The difference in concentration measured between the measurement and reference air stream, or before and after a measurement chamber, multiplied by the flow rate, provides the O_2 consumption or CO_2 production (turnover) of the bee in volume units per time interval (Equation 1). Table 1 provides a short reference of how to convert STPS volumes to moles or energy and power units.

$VCO2$ *or* $VO2$ = concentration × flow (ppm × ml min^{-1} × 10^{-3} = µl min^{-1}) (Equation 1)

6.1.1.2. Air drying, CO_2 scrubbing and tubing

1. *Chemical scrubbers:* Drying of the air by some means is a prerequisite to obtain accurate results in insect respiratory measurements. Fuel-cell O_2-measurement devices require completely dry air. Chemical scrubbers (desiccants) are inserted in columns, usually before the mass flow controllers. A commonly used desiccant is Drierite (contents: >98% $CaSO_4$ (gypsum), <2% $CoCl_2$). For special purposes, e.g. if CO_2 has also to be absorbed, granular magnesium perchlorate, $Mg(ClO_4)_2$, Ascarite is an excellent CO_2 scrubber.
2. *Cool traps:* For CO_2 measurement with a differential infrared gas analyser (DIRGA) complete air drying is not necessary in all cases. Cool traps (regulated at temperatures of ca. 2-10°C, for example) can bring relative humidity to a low and constant level. This works well because DIRGAs are typically operated at internal temperatures of >50°C. Relative humidity during measurement is therefore low. Since the water content reaching the DIRGA is the

Table 1. Quick reference for result calculations and units conversion. [#] molar volume of an ideal gas at 0°C and 101.32 kPa (760 Torr or 1 atm) of pressure; [##] 21.117/60, [###] caloric equivalent of sucrose (21.117 kJ l^{-1}, e.g. during sucrose feeding), use different values on demand (Table 2).

Desired results in units of VO2 or VCO2		**Calculate**
nl/min		ppm × ml/min
nmol/min		ppm × ml/min **/** 22.414[#]
Units conversion		
From	**To**	**Calculate**
µl/min	nMol/min	µl/min × 44.61497
nMol/min	µl/min	nMol/min × 0.022414
nMol/min	ml/h	nMol/min × 0.0013448
µl O_2/min	W (J s^{-1})	µl/min × 0.35195[##] × 10^{-3}
µl O_2	J	µl × 21.117[###] × 10^{-3}

same during calibration and measurement, and in the measurement and reference gas stream, differential measurements are accurate. However, independent cool traps are needed for the measurement and the reference gas streams (Lighton, 2008; Stabentheiner *et al.*, 2010), which makes the equipment more expensive.

3. *Tubing:* Tubing materials are diverse. A flexible and very durable material well suited for respiratory measurements is Viton®.

Specialists of insect respiration also use metal tubes (which of course are less flexible). Inner diameter of tubes should in any case be small, e.g. 2-4 mm, to achieve optimal results with honey bees.

User safety advice: Take care not to inhale Drierite. While fine gypsum particles may already impair respiratory functions, $CoCl_2$, which is used as a humidity indicator, is extremely hazardous. Ascarite is also extremely hazardous. To quote from the Oxzilla 2 (Sable Systems International) manual: "Try not to breath too much of it if you plan to see your grandchildren."

6.1.1.3. Serial measurement arrangement

Serial plumbing has the advantage that during differential measurement of CO_2 production or O_2 consumption both the measurement and the reference parts of the sensor devices always are operated at exactly the same flow rate. It is also cheaper than a parallel setup (see section 6.1.1.4.) because only one mass flow controller is needed (Lighton, 2008; Stabentheiner *et al.*, 2012).

1. *Air supply:* Fresh air should be taken from outside the laboratory via a tube to avoid disturbance of the measurements by air exhaled by the experimenters. The delay between the reference and measurement tubes or detectors of the measurement devices may otherwise lead to unnecessary baseline fluctuations. To additionally dampen fluctuations in outside air CO_2 or O_2 content, the air should be passed through a buffer container (5-10 l). An alternative is air from a compressed-air bottle.
2. *Pumps:* A pump feeds the air to a drying unit [e.g. Drierite columns (see safety advice in 6.1.1.2.), or a cool trap regulated at a low temperature of 2–10°C], which brings the water content of the air to zero or a low and constant level. Regulated pumps are of advantage because they help to avoid unnecessary overpressures in the system (offered, for example, by Sable Systems International).
3. *Mass flow regulation:* Subsequently, the air has to pass a mass flow controller (e.g. Brooks 5850S, Brooks Instruments; or Side Trak 840-L, Sierra Instruments; etc.). A range of 0–1000 ml/min is suitable for investigating both resting end endothermic honey bees. A small range mass flow controller of 0–100 ml/min may be recommended to improve accuracy of flow regulation for measurements of tiny honey bee larvae which may weigh considerably less than 1 mg in early stages of development (Petz *et al.*, 2004).
4. *Measurement:* The air then has to pass the reference sensor of the differential gas analyser. Afterwards, the air is put through the measurement (respirometer) chamber containing the honey bee (see section 6.1.1.5.). Before the air is forwarded to the measurement sensor it has to be dried again (and in the same way as the reference air stream).

6.1.1.4. Parallel measurement arrangement

In parallel measurement mode, two sets of pumps and mass flow controllers provide the reference and measurement sensors of the measurement device simultaneously with independent gas streams. This avoids delays between reference and measurement gas stream if the tubing of both sides has the same length. A well-tried sequence of devices and parts for the measurement stream is:

measurement/(reference) chamber(s) – drying columns – pumps – mass flow controllers – measurement device.

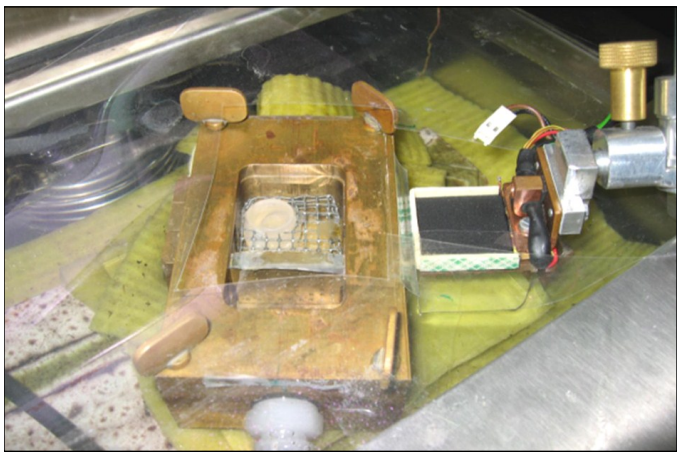

Fig. 9. Example of a measurement chamber for use in a temperature controlled water bath. It was milled out of a brass block. Air inlet visible in the picture bottom (front), outlet on top (covered by yellow kitchen cloth). Effective chamber volume is variable by a movable, perforated brass barrier. In this setup, the window in the lid was covered by an infrared transmissive plastic film allowing visual and thermographic behavioural observations and body surface temperature measurements (Kovac *et al.*, 2007; Stabentheiner *et al.*, 2012). Lid tightness was assured by a Viton® O-ring (not visible). Service holes were drilled in one side (left) to accept chromatography septa (11 mm), which allowed tight insertion of thermocouple wires. Tightness has to be proofed by applying an overpressure on the submerged chamber. The rectangle at the right-hand side is a proprietary reference radiator for infrared camera calibration (see Stabentheiner *et al.*, 2012).

Photo by Anton Stabentheiner

Inserting the columns for air drying before the pumps helps in this arrangement to considerably attenuate coupling of pump noise to the measurement chamber at the tube inlet (Stabentheiner *et al.*, 2012).

The parallel mode is preferable with fuel cell devices (for O_2 consumption measurement) because they are very sensitive to pressure differences between the reference and measurement channels (see section 6.1.2.1.).

The parallel mode is also preferable if the respiratory quotient (RQ, quotient VCO2/VO2) is to be determined with open-flow respirometry, with a combination of an infrared gas analyser (VCO2; section 6.1.3.) and a fuel-cell device (VO2; section 6.1.2.) in series. The need for frequent recalibration of fuel-cell instruments necessitates a possibility to bypass the bee measurement chamber. This can be done with manual three-way valves which allow smooth switching. If automated switching is intended magnetic valves are ***not*** suited because their pressure impulses lead to large unwanted signal offsets (Stabentheiner *et al.*, 2012). Motor stepping valves are be an alternative.

6.1.1.5. Measurement chambers

1. *Resting and active metabolism:* Fig. 9 shows a brass variant of a measurement chamber. For temperature control the chamber can be immersed in a water bath. If the chamber is not completely submerged in the water and the lid is exposed to the laboratory air in order to allow visual or thermographic observation of the bees (Fig. 9; Stabentheiner *et al.*, 2012) the air temperature in the chamber may differ from the water bath temperature. The strength and direction of this deviation depends on the difference between water bath and laboratory air temperature. The resulting temperature gradients inside the chamber require placement of a temperature sensor (see section 5.) inside the chamber. Completely submersing the chamber (and the inlet tubing) avoids this effect.
2. *Outdoor measurement:* A measurement chamber variant for outdoor measurements of foraging honey bees of about 8 ml inner volume was presented in Stabentheiner *et al.* (2012; Fig. 2 and Fig. 5 therein). Its lid could be opened and closed quickly to give the bees fast access to an artificial flower inside. It was also operated in serial mode. These measurement chambers can, of course, also be used in parallel mode. In cases where the sun shine is hits the measurement chamber, cooling via a water bath is indispensable, since the temperature inside can quickly reach critical levels and the bees will no longer enter it (Stabentheiner *et al.*, 2012).

In parallel mode, a different configuration is useful especially for field measurements (Stabentheiner *et al.*, 2012). A set of two identical measurement chambers can be placed at the air inlets of reference and measurement air streams, one for the measuring and the other for the reference air stream. These chambers can be constructed out of a small plastic film cylinder attached to a glass laboratory funnel. Attaching the base of the cylinder to an iron spacer ring as a counterpart of pieces of hard disk magnets fits the chamber to an underlying artificial flower. Air inlets are in the base of the artificial flower.

3. *Compensation of measurement gas loss:* Chamber opening may lead to an unwanted loss of measurement gas. This is especially critical when measuring foraging honey bees where the duration of stay at a food source can be shorter than a minute during unlimited feeding. In such cases as it is not possible to simply cut out a section of the measurement signal and to calculate an average over a certain time interval, special calibration is necessary. Very briefly, such a procedure compares the washout volumes from the chamber containing certain concentrations of CO_2 (or O_2) with and without chamber opening. Stabentheiner *et al.* (2012) provide a detailed description of how to compensate for such losses.
4. *Measurement of larvae and pupae:* For the measurement of tiny honey bee larvae, measurement chambers have to be very small (e.g. 0.5–2 ml). This can be realized by adapting a plastic syringe (Petz *et al.*, 2004), or with plastic photometer cuvets. For very young larvae and for eggs, the closed chamber method has to be used (see section 6.1.8.).

Table 2. Caloric equivalents (oxyjoule equivalents) and calorific values of biological substrates.

Substrate	Caloric equivalent (kJ lO_2^{-1})	Calorific value (kJ g^{-1})
Carbohydrates		
general mean	~21.15	~17.2
sucrose	21.117	16.8
glucose	21.0	15.6
Lipids	~19,6	~38,9
Proteins	~19,65	~17,2

5. *Flight metabolism:* The other extreme of chamber size is required in measurements of flight metabolism. For this purpose, large measurement chambers of about 0.3 to 1 l volume have to be used. Such chambers allow respiration measurements in agitated free flight (Harrison and Hall, 1993; Harrison *et al.*, 1996; Wolf *et al.*, 1996; Roberts and Harrison, 1999; Woods *et al.*, 2005). Measurement of bees in free hovering flight was accomplished in a wind tunnel by Wolf *et al.* (1989), which equals an even larger chamber size of about 4 l volume. Simulating an appropriate virtual environment to stimulate the bees to stay airborne for a longer time period without agitation is, however, a tricky task (Wolf *et al.*, 1989).
6. *Whole colony metabolism:* Even larger chamber volumes will be needed for whole colony measurements (Kronenberg and Heller, 1982; Southwick, 1985).

6.1.2. O_2 consumption

Flow-through oxygen measurement devices are of the fuel cell or paramagnetic type. For individual honey bees, fuel cell devices are in common use.

6.1.2.1. Fuel cell devices

From the different types of fuel cells, electro-galvanic lead-oxygen cells can be operated at room temperature (low temperature cells), while Zirconia cells require internal cell heating to high temperatures (> 500°C). Lead-oxygen cell devices (like the Oxzilla 2, Sable Systems; the S 104, Qubit Systems; etc.) are not extremely fast in reaction but appropriate for honey bee measurement and easy to handle. These cells are very sensitive to pressure differences between reference and measurement channel. Pressure sensitivity is an inevitable consequence of the fact that fuel cell devices do not measure O_2 'concentration' but O_2 'partial pressure'. Therefore, a parallel measurement setup is of great benefit (see section 6.1.1.4.). Their high sensitivity to (partial) pressure changes is, on the other hand, the basis of their high sensitivity. The slower (but usually sufficient) reaction of low temperature fuel cells may (in part) be compensated mathematically (post-hoc) by a $\mathcal{Z}$ transformation ("instantaneous correction") if restoration of the original shape of a respiratory event is an important parameter in an experiment (Lighton, 2008). Zirconia cells react faster. Appropriate air drying is indispensable in all fuel cells to achieve the best results.

6.1.2.2. Paramagnetic devices

Paramagnetic devices are not so sensitive but are well suited for continuous monitoring of colony oxygen consumption. Their sensitivity to tilting, however, requires installation in a firm position throughout an experiment.

6.1.2.3. Calibration

Calibration of fuel cell and paramagnetic devices is simple, usually just an end point calibration against outside air (20.95% O_2 content). Zero point calibration is usually not necessary. A certain drift, however, is inherent to the nature of fuel cells. Frequent recalibration and baselining during evaluation for drift compensation is, therefore, indispensable (Lighton, 2008).

6.1.2.4. Indirect calorimetry: calculation of energy turnover

Measurement of O_2 consumption (VO2) allows calculation of honey bee energy turnover (P, metabolic power) by multiplication with the caloric equivalent (oxyjoule equivalent, CE). This is called indirect calorimetry.

$$P\ (W) = VO2 \times CE \quad (lO_2\ s^{-1} \times J\ lO_2^{-1} = J\ s^{-1} = W) \text{ (Equation 2)}$$

The caloric equivalent depends on the substrate combusted in an animal's metabolism. In honey bees this is mainly carbohydrates (Rothe and Nachtigall, 1989). Table 2 summarizes some values of the caloric equivalent and of the calorific value of biological substrates.

6.1.3. CO_2 production

For the measurement of honey bee CO_2 production in open flow respirometry, a differential infrared gas analyser (DIRGA) is recommended. These measure infrared light absorption in the λ = 2.5 –8 µm wavelength range by the CO_2 present in air (photometric principle according to the Lambert/Beer law). These devices are very sensitive down to the sub-ppm range and usually offer a high baseline stability if their internal temperature is regulated accurately. Internal construction is mostly a set of two tubes (for reference and measurement signal) with an infrared lamp on one side and a detector on the other.

Portable instruments, originally designed for field measurements of plant photosynthesis, can also be used for field measurements of

honey bee respiration (e.g. Li-COR LI-6400XT Portable Photosynthesis System). This device also offers the possibility of simultaneous measurement of air H_2O content.

Fig. 10 shows a respiratory CO_2 trace of a honey bee. The bee was kept in the dark during the night, which made her soon entering the resting state. At rest she showed typical patterns of discontinuous respiration. At hive temperatures of 30°C or 34°C a bee breathes on average only once every 37 or 28 seconds, respectively (Kovac *et al.*, 2007). This trace, however, also shows the bees' high proneness to disturbance when caught in a respiratory chamber against their own will. When the experimenter entered the laboratory ('!' in Fig. 10) and afterwards switched on the light, the trace characteristic changed and the bee eventually heated up. Such endothermy increased energy turnover by more than the 100-fold.

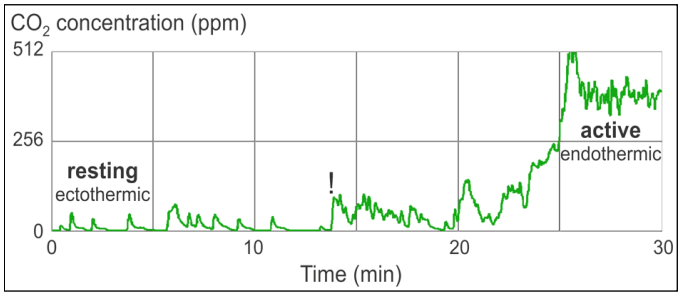

Fig. 10. Respiratory trace of a honey bee (*Apis mellifera carnica*). The bee was kept overnight in a 18 ml respiratory chamber (Fig. 10; 0 min = 07:21 in the morning). Left trace part: discontinuous gas exchange cycles during rest (light off), the bee was ectothermic; exclamation mark: experimenter entering the laboratory (with subsequent light on); right trace part: endothermy (bee prepared for immediate flight) and was actively seeking for an exit out of the chamber. Ambient temperature near the bee: ca. 22°C. For more resting traces see Kovac *et al.* (2007). Graph by Anton Stabentheiner

6.1.3.1. Measurement range selection

DIRGA measurement range and sensitivity is determined by the tube (or better: infrared beam) length. A frequently offered measurement range is 0–3000 ppm, well suited for the measurement of endothermic or flying bees.

0–250 ppm tubes in the DIRGA provide a high resolution, well suited for resting metabolism measurements of bees and for tiny honey bee larvae. With this measurement range, however, endothermy of adult bees will soon produce a range overflow. With a 0–10,000 or 0–20,000 ppm tube, the respiration of even whole colonies can be measured. A high resolution (small range) and a large range (lower resolution) system in series always provides a calibrated output (ABB, Inc.; Stabentheiner *et al.*, 2012).

It has to be considered, however, that there exists an interrelation between the gas flow and the height of the measurement signal (Gray and Bradley, 2006; see section 6.1.5.). In endothermic foragers (in an 8-18 ml measurement chamber) for example a flow rate of 250 ml

Table 3. Dependence of the respiratory quotient (RQ) of honey bee larvae on age and mass (*A. m. carnica*). * from Petz *et al.* (2004); ** from Melampy and Willis (1939).

age (h)	mean mass (mg)*	RQ**
12	0.36	
36	1.47	
60	10.2	1.42
84	37.73	1.23
108	131.44	1.23
132	159.66	1.13

min^{-1} provides a sufficient signal height with a 0-3000 ppm DIRGA. During investigations of honey bee resting metabolism with a 0–250 ppm tube, a flow of 150 ml min^{-1} turned out to provide a good compromise between sensitivity and temporal resolution of CO_2 production measurements in a wide range of ambient temperatures (2.5–45 °C; Kovac *et al.*, 2007). With small honey bee larvae, the flow rate has to be reduced, for example to 20 ml min^{-1} (Petz *et al.*, 2004).

6.1.3.2. DIRGA Calibration

Calibration of a DIRGA is usually performed against outside air at zero point (nowadays about 380 ppm CO_2; e.g. Petz *et al.*, 2004), and against a span gas of known CO_2 concentration in end point (according to the system's measurement range) or with internal calibration cuvettes (e.g. URAS devices of ABB, Inc.). Automatic calibration can be done after switching the air flow with magnetic (or better, stepping motor) valves to bypass the insect measurement chamber (see Stabentheiner *et al.*, 2012). The resolution of sensitive CO_2 measurement equipment is down to <0.2 ppm. With appropriate care, a measurement accuracy of ~2 ppm is feasible.

6.1.3.3. Respiratory quotient (RQ): calculation of energy turnover from CO_2 measurements

Since the respiratory quotient RQ = 1 in adult honey bees (Rothe and Nachtigall, 1989), energy turnover can be calculated directly from measurements of CO_2 production. In honey bee larvae, however, the situation is not just different but, so to say, unusual. Typically, animal RQ values are in the range of 1.0 for carbohydrate combustion, 0.8 for protein and 0.7 for pure lipid metabolism. If carbohydrates are converted to lipids, and during growth, RQ may be >1. In growing honey bee larvae, however, RQ values are not only >1, but also variable (Melampy and Willis, 1939; Table 3).

6.1.4. H_2O balance

In honey bees, there are several pathways of water loss, viz. cuticular transpiration, respiration, the mouthparts (for cooling), and defecation. Except for the latter, net water loss (and this way evaporative heat loss) can be readily measured with a DIRGA, simultaneously with CO_2 production (Roberts and Harrison, 1999).

6.1.5. Impact of flow control and measurement chamber size on sensitivity and temporal resolution

Gas flow has to be accurately regulated by mass flow controllers (compare section 6.1.1.3.). The respiratory output signal, however, depends on several factors (Gray and Bradley, 2006; Lighton, 2008; Terblanche *et al.*, 2010). Always consider that washout phenomena and signal delays influence measurements, depending on the volumes of the measurement chamber and the tubing. If you see respiratory peaks like in Figure 10, for example, keep in mind that signal rise and signal decay characteristics are always distorted. With proper calibrations and mathematical treatment (Z transformation; Lighton, 2008), the original signal characteristics may be restored to some extent for individual respiratory peaks or events.

Signal height is also influenced by several factors. In the first place it is influenced by the measurement chamber volume. Smaller volumes lead to a higher CO_2 accumulation and O_2 depletion. A low flow rate (recommended for small individuals) accumulates CO_2 (decreases O_2; Fig. 11) and therefore increases signal height. On the other hand, this reduces temporal resolution. A high flow rate decreases CO_2 concentration (increases O_2) and this way decreases signal height, but improves temporal resolution. A high flow rate may help to avoid signal overflow, for example if larger bee groups or whole colonies are to be measured. One has always to find a compromise between sensitivity and temporal resolution. Taking a measurement chamber as small as possible and increasing flow speed to a not too low value will increase sensitivity, and this way improve detection of small changes in the respiratory signal. A too high flow rate in a small chamber, however, will increase convective heat loss, especially in endothermic or flying bees. As thermoregulating bees probably counteract such additional losses, the measured metabolic rates may be overestimated as compared to natural situations.

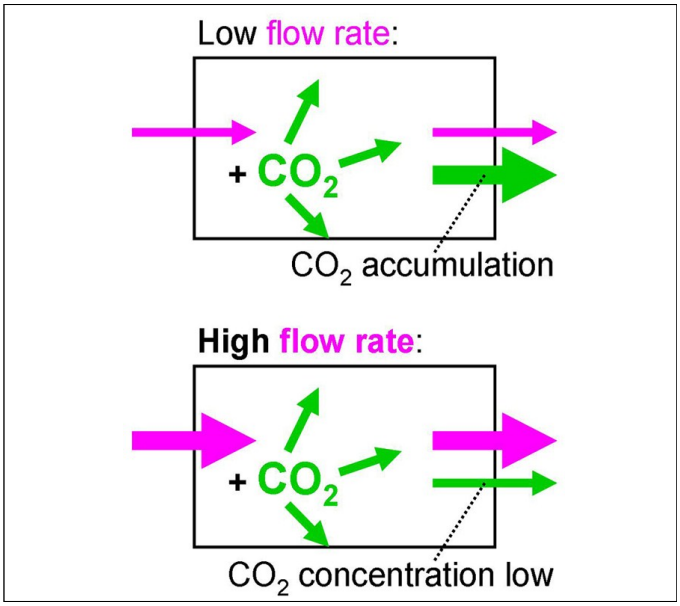

Fig. 11. Effect of flow rate on the respiratory measurement signal. Graph by Anton Stabentheiner

6.1.6. Controlling relative humidity

Desiccation is usually not a severe issue in endothermic bees because they produce much metabolic water (Roberts and Harrison, 1999). However, it may influence respiratory measurements of larvae or pupae. To avoid desiccation during longer lasting measurements (e.g. overnight), the relative humidity in the measurement chamber can be adjusted by saturating the air with water vapour by passing it through flasks with distilled water immersed in a temperature controlled water bath prior to the respirometer chamber. The temperature of this water bath is adjusted to the dew point temperature ($T_{dewpoint}$, °C) that corresponds to the desired relative humidity (rH_{des}, %) at the desired temperature inside the measurement chamber (T_{des}, °C) (Stabentheiner *et al.*, 2012):

$$T_{dewpoint} = 234.15 \times \ln(VP/6.1078) / [17.08085 - \ln(VP/6.1078)] \quad \text{(Equation 3)}$$

$$VP = rH_{des} \times SVP / 100 \quad \text{(Equation 4)}$$

$$SVP = 6.1078 \times \exp^{(17.08085 \times Tdes) / (234.175 + Tdes)} \quad \text{(Equation 5)}$$

where VP is vapour pressure (mbar), and SVP is the saturation vapour pressure (mbar).

6.1.7. Closed chamber method (CO_2 accumulation)

With honey bee eggs, flow through respirometry is not directly applicable, but this can be done with a closed chamber method. In a protocol described by Mackasmiel and Fell (2000), a measurement chamber (e.g. 2 ml autosampler vials) containing one egg on a piece of cell base wax is closed for some time (e.g. 12–14 h) to accumulate the CO_2 produced by the eggs. After certain periods, air samples of 250 µl are drawn from the vials and injected into an infrared gas analyser (IRGA). Accuracy of egg respiration measurement is ascertained by comparison with the air of empty vials and with the air of vials containing dead eggs (frozen overnight) or containing just a piece of cell base wax (Mackasmiel and Fell, 2000). To achieve a high accuracy of CO_2 measurement, the IRGA should be flushed with air deprived of CO_2 by pumping it through a calcium carbonate container. CO_2 can also be scrubbed with $Mg(ClO_4)_2$, or Ascarite (but see User Safety Advice for these substances in section 6.1.1.2.). It is also possible to consider a setup where the chamber is inserted in the gas path of a DIRGA and CO_2 accumulation is accomplished by closing magnetic valves for appropriate periods.

6.1.8. Thermolimit respirometry

Thermal limits of insects are often determined by visual or video observation (e.g. Hazell *et al.*, 2008; Hazell and Bale, 2011; Terblanche *et al.*, 2011; and citations therein). These protocols test the ability of coordinated movement of the insects. Besides passive observation, such analyses use the "righting response" of insects to regain normal body position after being pushed or nudged by the experimenter.

An alternative is thermolimit respirometry (Lighton and Turner, 2004; Lighton, 2008). With this method, the thermal limits of respiration can be determined. In *A. m. carnica,* chill coma temperature was determined by thermolimit respirometry to be in the range of 9-11°C by the observation that discontinuous respiration ceased (Lighton and Lovegrove, 1990; Kovac *et al.*, 2007; compare Hetz and Bradley, 2005). At these temperatures, muscular and neural functions of bees come to a halt (Esch, 1988). Honey bees can survive chill coma temperatures (<10°C) for a longer time, but are incapable of coordinated movement (Esch, 1988).

Thermolimit respirometry is especially useful in determining the upper critical thermal limit (CT_{max}). By applying a temperature ramp of 0.25°C min^{-1} (Terblanche *et al.*, 2011), the respiratory CT_{max} of *A. m. carnica* was determined as 48.9°C, which equalled the (voluntary) activity CT_{max} of 49.0°C (Käfer *et al.*, 2012). The method uses the fact that cyclic patterns of respiration cease suddenly at the CT_{max} (Lighton and Turner, 2004; Lighton, 2008; Käfer *et al.*, 2012).

In order to better identify the point of respiratory failure, the absolute difference sum (ADS) of the respiratory signal (rADS) can be calculated (Lighton and Turner, 2004). This is done by summing the difference between the absolute values (without considering sign) of successive data points of the respiratory signal. Further improvement can be achieved by calculating the rADS residuals around the estimated temperature of the CT_{max}. The rADS residuals are the difference between the ADS curve and a regression line calculated through the values for a section of, for example, 10 min before and after the estimated thermal limit. A usually sharp inflection point of the rADS residuals accurately indicates the respiratory CT_{max} (Lighton and Turner, 2004; Käfer *et al.*, 2012; Stabentheiner *et al.*, 2012). This method can also be applied for the analysis of other cyclic events like the readout of activity detectors (see section 6.8.2.).

One has to keep in mind, however, that thermolimit respirometry cannot replace conventional methods, which use behavioural cues like voluntary and forced activity to completely determine the CT_{max} and CT_{min}. Especially lethal temperatures have to be determined with appropriate methods of activity monitoring and proper tests of survival (e.g. Ono *et al.*, 1995; Ken *et al.*, 2005; Hazell *et al.*, 2010; Terblanche *et al.*, 2011). Nonetheless, thermolimit respirometry provides a standardized, fast and objective possibility to determine thermal limits because insect respiration depends on active control of spiracle and abdominal respiratory movements to achieve sufficient exchange of respiration gases. Consequently, respiration and muscular and neural activity are closely related. If respiration fails it is likely that other muscular and neural functions in the honey bee body are beyond their limits.

6.2. Chemical-optical oxygen sensors

Due to their small size chemical optical O_2 sensors allow measurement of O_2 concentration in very small volumes with a high spatial and temporal resolution (PreSens - Precision Sensing GmbH). For bees, these can be miniaturized chambers for use in metabolism measurement, including inside of honey bee brood cells. These oxygen microsensors are available with tip sizes of <50 µm. They consist of a silica fibre with a sensor spot at the tip which emits fluorescence when illuminated with the light of a blue LED through the fibre. Through a second (parallel) fibre this fluorescence signal is led back to a photodetector. If the sensor tip encounters an oxygen molecule, the excess light energy is transferred to the O_2 molecule, which decreases (quenches) the fluorescence. The degree of quenching correlates with the partial pressure of oxygen in the sensory material, which is in dynamic equilibrium with oxygen in the sample (e.g. surrounding air). With a pair of these sensors, one before and one after passage through the measurement chamber, it is possible to measure O_2 consumption in a flow-through setup. There are also sensors available for CO_2 measurement. At present, however, they only work in aqueous solutions.

6.3. Manometric and volumetric respirometry

From the variety of manometric and volumetric methods of O_2 consumption and CO_2 production measurement (Lighton, 2008), Warburg manometry is the most important one. It is a closed-system constant-volume method, which means that the measured O_2 concentration in the measurement vessel decreases in the course of an experiment. Therefore, air refreshment in regular intervals is necessary (Balderrama *et al.*, 1992; Stabentheiner *et al.*, 2003; Garedew *et al.*, 2004). This limits experimental time. Air drying is usually done with silica gel. CO_2 scrubbing can for example be done with caustic potash solution (KOH; see Lighton, 2008 for details). Moffatt and Núñez (1997) described a volumetric setup for the measurement of O_2 consumption of honey bees foraging from artificial flowers.

6.4. Titration methods

CO_2 production of foraging honey bees can also be determined by a titration method. By determining the amount of time an air current is needed to titrate 112 nanoequivalents of $Ba(OH)_2$, Moffatt (2000, 2001) determined the energy turnover of honey bees foraging from artificial flowers.

6.5. Isotopic tracer techniques

The doubly-labelled water technique is an appropriate method to measure field metabolic rates of free ranging insects (Wolf *et al.*, 1996; Speakman, 1997, 1998). It uses the differential elimination of ^{2}H and ^{18}O isotopes from the body tissues to determine metabolic rate. Note, these are stable isotopes and, thus, not radioactive. The principle is explained by Wolf *et al.* (1996) as "the fact that isotope concentrations decrease exponentially with time through the natural 'wash-out' of CO_2 and water. The hydrogen isotope is lost as water only, and the

oxygen isotope as both water and CO_2. Therefore, the apparent turnover rate of ^{18}O is higher than that of ^{2}H, and the difference between the two apparent turnover rates reflects the CO_2 production rate". Wolf *et al.* (1996) validated this method for bumble bees. They injected small volumes (1 µl) of a mixture containing ^{2}H and ^{18}O. After ~10 minutes, when the mixture had equilibrated with the body water pool, they withdrew 1–2 µl of haemolymph to determine the initial ^{18}O concentration. After 5–7 h, final blood isotope content was analysed by mass spectrometry. This technique actually provides rates of CO_2 production, which can be directly used to calculate energy turnover of honey bees, because their RQ = 1 (Rothe and Nachtigall, 1989).

6.6. Calorimetry

Calorimetry allows the direct measurement of heat production (energy turnover) of individual bees or bee groups (Fahrenholz *et al.*, 1989, 1992). Differential calorimeters, constructed as twin-setups of 'camping box calorimeters' are the most suited variants for honey bee (and insect) calorimetry (e.g. Fahrenholz *et al.*, 1989; Lamprecht and Schmolz, 2000). Depending on construction size, measurements are possible on individual honey bee larvae (Schmolz and Lamprecht, 2000) and adult bees (Schmolz *et al.*, 2002), in whole colonies (Fahrenholz *et al.*, 1992), and even in groups of tiny honey bee parasites (*Varroa destructor*, Garedew *et al.*, 2004). The insects inside these instruments can be continuously supplied with fresh air if necessary, which allows long duration measurements (Schmolz and Lamprecht, 2000; Schmolz *et al.*, 2002).

Construction of such a calorimeter is not difficult ("poor man's calorimeters"; Lamprecht and Schmolz, 2000; see also Lighton, 2008). Peltier units are attached to the bottom of camping boxes to keep the instrument at a desired temperature. It is an inherent property of Peltier elements that heating or cooling of one side can be regulated via the direction of the current applied.

To sense heat flux, such constructions use the fact that Peltier elements can also be used as thermopile heat flux sensors, because a temperature gradient along them produces a current (Seebeck effect). Such boxes are commercially available with variable volumes (~5–30 l). Heat flux sensitivity is about 10±30 mV/W, which is in the range of commercially available instruments (Lamprecht and Schmolz, 2000). To achieve an acceptable baseline stability, a differential (twin) setup is recommended, with one box serving as (empty) reference unit. An additional styrofoam insulation around both boxes compensates for an otherwise poor baseline stability.

A frequent disadvantage of calorimeters is their large time constant (see Lamprecht and Schmolz, 2000; Lighton, 2008). This means that the output signal of heat production may be heavily distorted, hiding short-time dynamic properties of events of heat production or heat release. Appropriate calibration and signal processing may help to compensate for such shortcomings and to restore the dynamic structure of the signal (see pp. 74-75 in Lighton, 2008).

6.7. Energetics derived from measurement of sugar consumption

Honey bees fuel their flight nearly exclusively with sugars (Rothe and Nachtigall, 1989). This provides the opportunity to calculate flight energy consumption from the determination of sugar consumption (Hrassnigg *et al.*, 2005; Brodschneider *et al.*, 2009; see also the *BEEBOOK* paper on behavioural methods (Scheiner *et al.*, 2013)). Usually such experiments are performed in tethered flight in a roundabout or in a wind tunnel. While such measurements are very well suited to answer certain questions of energy metabolism, it has always to be kept in mind that tethered flying bees do not bear their own weight. Calculated absolute values of energy turnover are, therefore, not a full representation of a natural flight situation.

6.8. Activity monitoring:

Energetic considerations and detailed investigation of honey bee respiration often require judgement of activity and behaviour.

6.8.1. Video and thermography:

The most direct method of activity monitoring is direct observation of behaviour and classification according to a (predefined) list of behaviours or stages of activity (Stabentheiner *et al.*, 2003a,b). This can be combined with video analysis. If it is to judge whether or not bees are at rest, infrared video analysis with an active infrared light source allows observation in the dark. Infrared thermography provides the opportunity to simultaneously observe the bees' behaviour in complete darkness and measure their body surface temperature.

6.8.2. Optical activity detectors:

Optical activity detectors, which use an infrared light source (LED) and a photodetector (photodiode, phototransistor, etc.) can monitor changes of the position of bees in the measurement chamber. The information on bee activity is reflected in fluctuations of the signal recorded by the photodetector. According to Lighton (2008), "the one challenge is to create an activity data channel that is easily interpreted." Among other possibilities, a possible approach is to sum the difference between absolute values (not considering sign) of successive data points of the activity signal. The resulting absolute difference sum (aADS; Lighton and Turner, 2004) shows a steep slope in phases of high activity. A clear breakpoint emanates if activity ceases. This method can be enhanced by calculating the aADS residuals (compare section 6.1.9.). It is considered as 'semi-quantitative'. A detector ready for use can be purchased at Sable Systems International.

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