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Sample preservation, transport and processing strategies for honeybee RNA extraction: Influence on RNA yield, quality, target quantification and data normalization

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ABSTRACT

Viral infections in managed honey bees are numerous, and most of them are caused by viruses with an RNA genome. Since RNA degrades rapidly, appropriate sample management and RNA extraction methods are imperative to get high quality RNA for downstream assays. This study evaluated the effect of various sampling-transport scenarios (combinations of temperature, RNA stabilizers, and duration) of transport on six RNA quality parameters; yield, purity, integrity, cDNA synthesis efficiency, target detection and quantification. The use of water and extraction buffer were also compared for a primary bee tissue homogenate prior to RNA extraction. The strategy least affected by time was preservation of samples at −80 °C. All other regimens turned out to be poor alternatives unless the samples were frozen or processed within 24 h. Chemical stabilizers have the greatest impact on RNA quality and adding an extra homogenization step (a QIAshredder™ homogenizer) to the extraction protocol significantly improves the RNA yield and chemical purity. This study confirms that RIN values (RNA Integrity Number), should be used cautiously with bee RNA. Using water for the primary homogenate has no negative effect on RNA quality as long as this step is no longer than 15 min.

1. Introduction

The European honey bee (*Apis mellifera*) is affected by numerous viral infections. At least 18 honey bee viruses has been identified, the vast majority being positive-sense single-stranded RNA viruses (Ribière et al., 2008). Despite enormous progress in the development of molecular nucleic acid-based assays for honeybee virus diagnosis (Evans et al., 2013), very little research has gone into optimizing the collection of bee samples from the field for subsequent RNA analysis (Chen et al., 2007; Dainat et al., 2011; Tentcheva et al., 2006).

RNA-based analyses such as real-time quantitative RT-PCR generally require collection of fresh samples and high-quality RNA. The goal of any RNA isolation procedure is to faithfully mirror the biomolecular status of the sample at the time for collection. However, RNA is rapidly digested by ubiquitous, potent and highly stable RNases. As a result, shorter fragments of RNA occur in the sample which may compromise the results of downstream assays (Fleige and Pfaffl, 2006). The quality and quantity of RNA is dependent on the RNA extraction methods, and in particular on the sample management strategy. Many parameters such as sample type, transportation time, temperature and chemical stabilizers may influence the final quality of the RNA. RNA degradation can be minimized by cold temperatures, desiccation, chemical preservatives or stabilizers to inactivate RNases, and not least by rapid processing. After sampling and extraction, certain criteria must be fulfilled to assure RNA quality (defined as the composition of purity and integrity) for downstream applications. The RNA should ideally be free of protein, free of genomic DNA, un-degraded, free of inhibitors for RT and PCR reactions and free of nucleases (Fleige and Pfaffl, 2006).

The purity of RNA can be determined by photometry, using spectrophotometers like the NanoDrop™ (Thermo Scientific). The ratio of absorbance at 260 nm (nucleic acids), 280 nm (proteins) and 230 nm (phenolics) is used to assess the purity of nucleic acids. Absorbance ratios A^{260}/A^{280} and A^{260}/A^{230} of > 1.8 is regarded as highly pure and generally accepted as suitable for gene expression studies (Fleige and Pfaffl, 2006).

The integrity of extracted RNA has historically been assessed by gel electrophoresis and subsequent analysis of the 28S and/or the 18S ribosomal RNA bands (Sambrook and Russel, 2001). This method is based on subjective human interpretation, but recently instruments have been designed to visualise and quantify the extent of RNA degradation, based on algorithms comparing various aspects of the ribosomal and non-ribosomal (mRNA) fractions in a trace profile. The

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result is represented numerically relative to pre-determined benchmark values (Schroeder et al., 2006). For example, the Bioanalyzer (Agilent Technologies, Waldbronn, Germany) is a sensitive and highly reproducible device for RNA quality control that allows the calculation of an RNA integrity number (RIN). A RIN value of 1 represents a fragmented and degraded RNA whereas a value of 10 represents intact and non-fragmented RNA (Schroeder et al., 2006). However, the RNA profile of insects differs significantly from the standard benchmarks, which are generally based on human and mouse samples, so the resulting suboptimal RIN values may easily be misinterpreted as degradation. Unfortunately, there is a general lack of awareness of the different rRNA composition in insects, including *A. mellifera*, which has led to poor interpretation of their rRNA profiles both in daily experiments as well as in publications (Winnebeck et al., 2010). In this study, RIN values as well as ribosomal values were correlated to real-time PCR data.

Many types of material can be sampled from a honey bee colony. Since whole bees cannot be adequately preserved in aqueous media (due to the lack of penetration of the exoskeleton), whole adult bee samples have limited storage options. These options increase when the bees are crushed, or when only part of the bees is sampled. Only live bees can really be sampled at ambient temperatures for long periods, but bees crushed on-site and in RNAlater or other stabilisers is another possibility. All viruses and most other pathogens can be found in the bee alimentary canal (Chen et al., 2006), and it is relatively easy to extract the guts from adult bees and even easier to squeeze out faecal contents. The guts also often have the highest virus titres so that there is little difference between sampling whole bees and just the gut contents (Chen et al., 2006). Such samples are particularly useful for individual bees. Entire guts can be stored in RNAlater or on FTA® cards (Whatman) and recovered intact for extraction. However, it is important to establish that such partial tissues are indeed representative of the bee as a whole, and in particular that the pathogen content of the guts can be related to infection of the other bee tissues.

In order to systematically explore how sample management affects RNA quality and the performance of RT-qPCR assays for honeybee samples, two experiments were conducted; a **Transport experiment** and a **Homogenate experiment**. The **Transport experiment** concerned various sample transport scenarios using different combinations of temperature control and RNA stabilizers, and investigated six RNA quality parameters; i) total yield, ii) purity, iii) integrity, iv) cDNA synthesis efficiency, v) RT-qPCR detection sensitivity (amplification of two internal reference genes), vi) quantification (amplification of two honeybee virus targets). The **Homogenate experiment** was set up to determine if the use of water rather than extraction buffer for preparing primary homogenates from bulk samples of adult bees affects the RNA quality, quantity and performance in RT-qPCR. The same parameters as in the first experiment were investigated, except iii) integrity.

2. Material and methods

2.1. Sample origins

For the **Transport experiment**, adult worker bees were collected from *A. mellifera* colonies in the apiary at the Swedish University of Agricultural Sciences in Uppsala, Sweden. The different sampling-transport scenarios used for this experiment can be found in Table 1. Estimates were made for the time taken to prepare the samples and the aggregate costs of transportation at different temperatures and with different preservatives (Supplementary Fig. 1). Eight identical samples containing 10 adult bees each were prepared for each regime. Two samples were analysed independently at four different time points, mimicking different durations of transport: same-day processing (0 days), 1 day, 4 days and 7 days after collection.

In the **Homogenate experiment**, a bulk sample (N = 50) of fresh adult bees was flash-frozen with liquid nitrogen and ground to a fine powder using a pestle. Half of this powder was then mixed with 5 ml (200 µl per bee) water and the other half was mixed with 5 ml of RLT buffer. Both homogenates were left at ambient temperature and duplicate 100 µl aliquots were removed from each after 0, 5, 15 and 60 min, for immediate RNA extraction.

Table 1
Summary of the sample preservation and transport conditions investigated.

Regime	Description
#1	10 Live bees transported in queen cages with candy, ambient temperature
#2	10 Whole bees, frozen in −80 freezer
#3	10 Live bees, transported on ice
#4	10 Whole bees in 10 ml 100% ethanol, transported on ice
#5	10 Crushed bees in 10 ml saturated ammonium sulphate, transported on ice
#6	10 Crushed bees in 10 ml RNAlater (Qiagen), transported on ice
#7	10 Crushed bees in 10 ml RNAlater (Qiagen), transported at ambient temperature
#8	5 Bee guts in 2,5 ml RNAlater (Qiagen), transported on ice
#9	5 Bee guts in 2,5 ml RNAlater (Qiagen), transported at ambient temperature
#10	5 Bee guts dried on FTA cards (Whatman), transported on ice
#11	5 Bee guts dried on FTA cards (Whatman), transported at ambient temperature

2.2. RNA extraction

In the **Transport experiment**, for the samples involving whole bees (intact or crushed), the 10 bees were put in a plastic mesh bag (BioReba) together with 2 ml nuclease-free water, flash-frozen in liquid nitrogen and ground to a fine powder using a pestle. Once the powder was thawed, 100 µl of homogenate was recovered from beyond the mesh and was mixed with 350 µl RLT RNA extraction buffer (Qiagen, Hilden, Germany). For the bee gut samples stored in RNAlater, the 5 bee guts were put together in a microfuge tube with 500 µl nuclease-free water and ground using a micro-pestle. The tubes were centrifuged briefly and 100 µl of the crude extract was mixed with 350 µl RLT buffer (Qiagen). For the bee gut samples stored on FTA cards, the dried bee guts were cut out from the FTA cards and soaked in 500 µl of nuclease-free water for 10 min with intermittent vortexing (as recommended by the manufacturer) after which 100 µl of the supernatant was mixed with 350 µl RLT buffer (Qiagen). Total RNA was extracted from the samples by a QiaCube robot using the Plant RNEasy™ protocol (Qiagen), with half of the samples including a QIAshredder™ step and the other half excluding the QIAshredder™ step. Included in each extraction run was a 'blank' extraction, as part of the quality control of the QiaCube. The RNA was eluted in 50 µl RNase-free water and stored at −80 °C until further use.

In the **Homogenate experiment**, the 100 µl aliquots collected at different time-points were mixed with 350 µl RLT buffer (Qiagen). Total RNA was extracted from the different extract/RLT buffer mixtures by a QiaCube robot using the Plant RNEasy™ protocol (Qiagen). Included in each extraction run was a 'blank' extraction, as part of the quality control of the QiaCube. The RNA was eluted in 50 µl nuclease-free water and stored at −80 °C until further use.

2.3. RNA yield, purity and integrity

Total RNA yield was determined by absorbance at 260 nm and the RNA purity was determined by the ratios A^{260}/A^{280} (nucleic acids/proteins) and A^{260}/A^{230} (nucleic acids/phenolics), using a NanoDrop™ 1000 Spectrophotometer (Fisher Scientific). The RNA Relative Integrity Number (RIN) was determined by microfluidic capillary electrophoresis using a 2100 Bioanalyzer (Agilent Technologies) and the default RNA profiles.