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*Review Paper*

# Cell Lines for Honey Bee Virus Research

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**Abstract:** With ongoing colony losses driven in part by the Varroa mite and the associated exacerbation of virus load, there is an urgent need to protect honey bees (*Apis mellifera*) from fatal levels of virus infection and from nontarget effects of insecticides used in agricultural settings. A continuously replicating cell line derived from the honey bee would provide a valuable tool for study of molecular mechanisms of virus – host interaction, for screening of antiviral agents for potential use within the hive, and for assessment of the risk of current and candidate insecticides to the honey bee. However, the establishment of a continuously replicating, honey bee cell line has proved challenging. Here we provide an overview of attempts to establish primary and continuously replicating hymenopteran cell lines, methods (including recent results) for establishing honey bee cell lines, challenges associated with the presence of latent viruses (especially *Deformed wing virus*), in established cell lines and methods to establish virus-free cell lines. We also describe the potential use of honey bee cell lines in conjunction with infectious clones of honey bee viruses for examination of fundamental virology.

**Keywords:** honey bee virus; Hymenoptera; insect cell culture; cell lines; Apis mellifera; Deformed wing virus

## 1. Introduction

About one third of all agricultural crops are dependent on the honey bee (*Apis mellifera*) for pollination, reflecting the importance of the honey bee to agricultural production. However, honey bee colonies in the northern hemisphere have been in decline [1-5]. With an estimated 59% total loss of colonies between 1947 and 2005 [1] and an average 40% annual loss of colonies from 2010 to 2016 [6-10], these declines are of ongoing concern [11]. While the causes of honey bee colony decline are complex [2], the ectoparasitic mite, *Varroa destructor*, represents a major threat to honey bee health [12, 13]. In addition to weakening honey bees by feeding on fat body [14], the Varroa mite also vectors honey bee viruses [15-20], with the spread of the Varroa mite resulting in dominance of a more pathogenic *Deformed wing virus* (DWV) strain [16, 21]. At least 24 honey bee-associated viruses have been reported [22], including seven viruses that are widespread. These are *Acute bee paralysis virus* (ABPV), DWV, *Sacbrood virus* (SBV), *Black queen cell virus* (BQCV), *Israeli acute paralysis virus* (IAPV), *Chronic bee paralysis virus* (CBPV), and *Kashmir bee virus* (KBV) [22, 23].

Insect-derived cell lines provide valuable tools for the study of insect viruses under controlled conditions, with their genetic uniformity providing for more consistent results. Cell lines may allow for the study of suborganismal processes that may not be tractable using the host organism. Insect cell lines can also be used for screening of insecticides or biocontrol compounds against pests, or for assessment of potential risk to non-target organisms such as the honey bee [24]. Approximately 1000

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insect-derived cell lines have been established according to the ExPASy Cellosaurus database with >80% derived from Diptera and Lepidoptera [25]. However, relatively few cell lines are derived from Hymenoptera.

A honey bee-derived cell line would provide a valuable tool for the study of virus-insect and virus-virus interactions. In this review, we provide a summary of establishment of primary cultures and continuously replicating hymenopteran cell lines, virus studies using the *Apis mellifera*-derived AmE-711 cell line, methods for establishment of virus-free cell lines, and potential applications of these cell lines in insect virology. A honey bee cell line would provide a powerful research platform for increased understanding of honey bee virology.

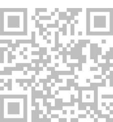
## 2. Establishment of hymenopteran cell lines

### *2.1 Primary cell lines*

A primary cell line is a cell line derived from specific insect tissues or organs, cultured on artificial medium and maintained for a limited time. Primary cell cultures have been established from three hymenopteran species including an ant (*Pseudomyrmex triplarinus*), a parasitic wasp (*Mormoniella vitripennis*) and the honey bee (*A. mellifera*) (Table 1) [26-29]. The longevity of these primary cell cultures was highly variable. Primary cell cultures derived from ant venom gland cells were maintained for up to 12 months while honey bee primary cell cultures were viable from days to months [26-28]. Most of the early primary cell cultures from the honey bee were derived from neural tissues (Table 1)[30-40]. An early primary neuron-derived culture, dissociated by mechanical treatment and prepared from specific regions of the pupal brain, survived for only three weeks [31]. Importantly, the cultured neurons showed surface properties and a transmitter phenotype similar to those of their *in vivo* counterparts [41], indicating the potential for primary cell cultures in the study of cell biology. Additional honey bee primary cell lines were established from eggs [42-45], guts [41, 46] and larval or pupal tissues (Table 1) [27, 35, 41, 47-49].

Similar procedures were used for generation of these primary cell cultures, as follows [28]. 1) Bees or tissues were surface sterilized using a sterilization buffer containing ethanol, hypochlorite or $H_2O_2$, and rinsed several times. 2) The tissue was gently homogenized or torn apart in a specific growth medium (e.g. L-15 cell culture medium, originally established for mammalian cell culture) with several types of antibiotics (e.g. gentamycin, penicillin, streptomycin), and an antimycotic (e.g. amphotericin B). Mechanical methods are typically used for establishment of honey bee primary cell lines as enzymatic dissociation of tissues resulted in limited numbers of isolated cells and contamination [41]. 3) The homogenate was transferred to an incubator with medium replaced at intervals until the expected morphology of the cells was observed. Primary cell types may be adherent or non-adherent (floating). 4) The identity of the cells was confirmed by polymerase chain reaction (PCR) amplification of a specific gene sequence from DNA extracted from cultured cells, and sequencing of the PCR product. Target genes included *actin* and *laminin* for confirmation of honey bee cell lines [43, 47]. Mitochondrial *cytochrome c oxidase subunit I* (*COI*) is also commonly employed for this purpose.

The cell culture medium used significantly influences cell growth rates, suggesting that specific nutrients are required for maintenance of honey bee cells. Media that support the growth of cell lines derived from other insects are mostly insufficient for maintenance of honey bee-derived cells. Evaluation of different media for cell growth is required, with cells growing extremely slowly in an



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85 unsuitable environment. For example, primary cells of *A. mellifera* were reported to show attachment
86 and growth in WH2, a medium modified from HH-70 psyllid culture medium, while they grew
87 slowly in two commercial media, Sf-900™III SFM and EX-CELL 405 [47]. Chan *et al.* (2010) transduced
88 bee cells using lentivirus, illustrating the use of molecular manipulations for developing immortal
89 cell lines. In this study, insect cell culture media (Grace's and Schneider's) and mammalian cell
90 culture media were compared with the former resulting in higher viability. Cryopreservation of bee
91 cells was also demonstrated for short-term storage. Two media were recommended (BM3 and L-15)
92 by Genersch *et al.* (2013) for the isolation and cultivation of neuronal cells from pupae or adults, and
93 gut cells from pupae [28]. Ju and Ghil used L-15 medium-based honey bee cell (LHB) growth medium
94 and Schneider's insect medium-based honey bee cell (SHB) growth medium with more cells
95 produced in the LHB medium than in SHB medium after six passages. The doubling time in LHB
96 medium was only about eight days [43]. Clearly, identification of a suitable cell culture medium is
97 critical for maintenance of primary cell cultures.

98 **Table 1.** Primary cell cultures from hymenopteran species.

| Species | Tissue | Longevity | Medium | Incubation | Year | Ref |
|---|---|---|---|---|---|---|
| *Pseudomyrmex triplarinus* | Venom glands | 1 year | PTM-1CC | 28 °C | 1985 | [26] |
| *Apis mellifera* | Antennal lobes | ~1 month | 5+4 and A2 | 29 °C | 1991 | [30] |
| *Apis mellifera* | Pupal honey bee brain | Three weeks | L-15 | 29 °C | 1992 | [31] |
| *Mormoniella vitripennis* | Eggs | 3 months | Grace | 28 °C | 1993 | [29] |
| *Apis mellifera* | Mushroom body | NA | L-15 | NA | 1994 | [32] |
| *Apis mellifera* | Kenyon cells | Up to 10 days | L-15 | 29°C | 1994 | [33] |
| *Apis mellifera* | Antennal lobe | NA | 5+4 | NA | 1994 | [34] |
| *Apis mellifera* | Antennal flagella | Several weeks | 5+4 | 30 °C | 1994 | [35] |
| *Apis mellifera* | Kenyon cells | Up to 6 weeks | L-15 | 26 °C | 1999 | [36] |
| *Apis mellifera* | Antennal motor neurons | NA | L-15 | 28 °C | 1999 | [37] |
| *Apis mellifera* | Kenyon cells and projection neurons | NA | L-15 | 26 °C | 2003 | [38] |
| *Apis mellifera* | Mushroom bodies neuroblasts | NA | L-15 | 26 °C | 2003 | [39] |
| *Apis mellifera* | Antennal lobes | ~1 month | L-15 | 26 °C | 2008 | [40] |
| *Apis mellifera* | Pre-gastrula stage embryos | More than 3 months | Grace | 30 °C | 2006 | [44] |
| *Apis mellifera* | Eggs | Four months | Grace's or Schneider's | 32 °C with 5% CO2 | 2010 | [45] |
| *Apis mellifera* | Pupae | At least 8 days | WH2 | 22 °C | 2010 | [47] |
| *Apis mellifera* | Gut | At least 6 days | L-15 | 33 °C | 2012 | [46] |
| *Apis mellifera* | Midgut | 15 days | WH2 | 27 °C | 2012 | [41] |
| *Apis mellifera* | Eggs | ~135 day | L-15 | 30 °C | 2015 | [43] |

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*2.2 Continuous cell lines derived from Hymenoptera*

A continuous cell line is a cell line comprised of a single cell type that can be passaged in culture for many generations or indefinitely [50]. In the Class Insecta, many well-characterized cell lines derived from Lepidoptera and Diptera have been described [25, 51, 52]. However, relatively few continuous insect cell lines from Hymenoptera have been reported (Table 2). These include cell lines derived from *Neodiprion lecontei* (Diprionidae)[53], *Trichogramma pretiosum* (Trichogrammatidae) [54], *T. confusum*, *T. exiguum* [55] and *Hyposoter didymator* (Ichneumonidae) [56] (Table 2). To our knowledge, replication of honey bee viruses in these cell lines has not been tested.

**Table 2.** Permanent cell lines derived from hymenopteran species.

| Species | Stage | Medium | Outcome | Year | Reference |
|---|---|---|---|---|---|
| ***Neodiprion lecontei*** | Embryos | Supplemented Grace's | 10 cell lines | 1981 | [53] |
| ***Trichogramma pretiosum*** | Embryos | IPL-52B + IPL-76 (3:1) | 1 cell line | 1986 | [54] |
| ***Trichogramma confusum*** | Embryos | modified IPL-52B | 1 cell line | 1991 | [55] |
| ***Trichogramma exiguum*** | Embryos | modified IPL-52B | 1 cell line | 1991 | [55] |
| ***Hyposter didymator*** | Pupae | HdM medium | 4 cell lines | 2004 | [56] |
| ***Apis mellifera*** | Larvae | Supplemented Grace's | 1 cell line (with c-myc gene) | 2011 | [57] |
| ***Apis mellifera*** | Embryos | HB-1 (modified L-15) | 1 cell line | 2013 | [58] |

The establishment of a continuous cell line from the honey bee has proven difficult with only two continuous cell lines reported (Table 2). Bergem *et al.* investigated the long-term maintenance of honey bee cells by generating cell cultures derived from different honey bee tissues and testing several culture media. Cell cultures were initiated from a specific stage of the honey bee embryo, the pre-gastrula stage, and cells remained mitotically active for more than three months [44], suggesting that honey bee embryos at this specific stage provide good starting material for long-term cultivation. Kitagishi Y *et al.* engineered *A. mellifera* cells derived from honey bee embryos using the human c-myc proto-oncogene for their long-term cultivation [57]. The cell line, designated as MYN9, was successfully cultured for more than 100 generations over a period of more than 8 months, suggesting human c-myc proto-oncogene was efficient for immortalization of honey bee cells. Honey bee marker genes and c-myc were detectable by PCR. However, the honey bee virus, *Deformed wing virus* (DWV) was also detected in the MYN9 cell line. While MYN9 was a honey bee-derived cell line, whether expression of c-myc in the cells affected endogenous gene expression is unknown.

A honey bee cell line derived from embryonic tissues, named AmE-711 (*Apis mellifera* cell line from Embryonic tissues, established on 7/2011), was reported by Goblirsch M. *et al.* [58, 59]. Similarly, mid to late stage honey bee eggs were used as the initial material for establishment of primary cultures as undifferentiated embryonic cells are continuously dividing. The AmE-711 cell line was isolated from one of multiple primary cell lines. Several challenges were encountered during the establishment of the AmE-711 cell line: 1) It took time for the honey bee cells to adapt to culture as

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128 most of the primary cultures required three months to reach confluence [58]; 2) Only one out of ~100
129 subsequent cell passages from primary cell cultures continued to replicate [58]; 3) The length of time
130 used for enzymatic treatment significantly influenced cell fate. Incubation with trypsin for more than
131 10 min lead to failure of cell re-attachment or cell injury [58].
132 The AmE-711 cell line contained bipolar and multipolar fibroblastic cells, elongated in shape
133 with an adherent growth phenotype. Most cells had a diploid karyotype, similar to honey bee cells
134 in nature. Most importantly, the cell line was continuous as it was maintained long term and
135 passaged at least 18 times with a minimum of 43 generations [58, 60]. However, the AmE-711 cell line
136 proved difficult to maintain and crashed in 2015 possibly due to virus infection (see Section 3. below).
137 Fortunately, this cell line has since been recovered and adapted to commercially-available medium
138 (Dr. Michael Goblirsch, USDA, ARS; personal communication).

139 *2.3 A systematic iterative protocol to establish tissue-derived insect cell lines from honey bees and other*
140 *challenging insect species: Recent results from BCIRL*

141 Hundreds of insect cell lines have been established since the first ones were produced in the late
142 1950's and '60's. [61, 62]. Some of these lines are in routine use in industry, university and government
143 laboratories. The Biological Control of Insects Research Laboratory (BCIRL) has a history of
144 establishing cell lines [63-68], generally using a standard protocol. This protocol has a core set of steps
145 systematically repeated with observation-based changes in media components that ultimately leads
146 to established, functional cell lines. A suitable medium based on experience and the literature is
147 selected for the first cell line initiation. In later iterations, cell lines are initiated with other media, and
148 sometimes with new media created by mixing known media or by adding media supplements. This
149 iterative process generally leads to the establishment of permanent cell lines useful in several research
150 and development programs [65, 67, 68].
151 In recent years we have been working to establish cell lines from honey bees at BCIRL. The
152 establishment of cell lines derived from honey bees has proven to be very difficult, similar to the
153 situation for a large group of insects from a variety of orders. It is not clear why cell lines are routinely
154 established from some orders of insects, such as Lepidoptera, but not others. Such differences in cell
155 line establishment may relate to fundamental cellular biology. We plan to investigate the point in
156 detail by tracing gene expression patterns during the establishment process using cell lines from
157 lepidopterans and coleopterans that are routinely established, and from recalcitrant species, similar
158 to work in *Drosophila melanogaster* cell lines [69].
159 All bee stages have been used for culture initiations, including adult workers and queens and
160 specific tissues within the bees. We have initiated lines with midgut, nervous system (ventral nerve
161 cord, brain, or both), aorta, fat body, ovaries, spermatheca, a combination of testes/fat body, muscle,
162 Malpighian tubules, venom sack, and ground pupal heads.
163 Cell culture initiations are performed in biosafety hoods with surface sterilized dissecting
164 implements (Fig. 1). Before dissection, the bees are immobilized in 70% ethanol (1 min) and surface
165 sterilized in a series of treatments, 0.8% sodium hypochlorite (2-3 min), 70% ethanol (3-5 min) and
166 rinsed 2-7 times in Hanks balanced salt solution (HBSS) or calcium, magnesium free – phosphate
167 buffered saline (CMF-PBS). Bees are pinned dorsal side up and an incision is made through the thorax
168 and abdomen. The opening is flushed with HBSS containing antibiotics (0.1 mg/mL gentamycin, 0.5
169 µg/mL amphotericin B and/or 50-200 U/mL penicillin, 0.05-0.2 mg/mL streptomycin, Millipore Sigma)

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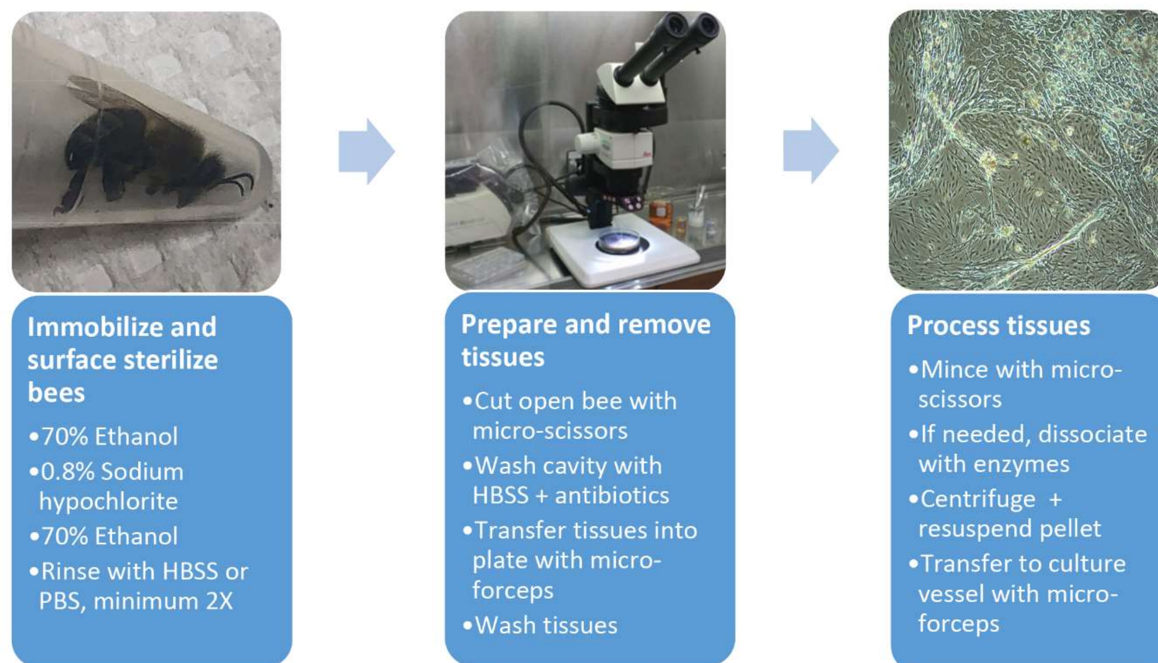
170 and selected tissues are removed with sterilized micro-forceps, washed three times in HBSS, and
171 collected in wells of a standard 24-well tissue culture plate. Tissues are minced with sterilized micro-
172 scissors, centrifuged if needed (800xg, 5 min, 4°C), then transferred into either tissue culture plates
173 (12-, 24-, or 48-well) or flasks ($T_{12.5}$, $T_{25}$) using cell culture media augmented with selected antibiotics
174 (50-200 U/mL penicillin, 0.05-0.2 mg/mL streptomycin). In some initiations, 0.5 ml of an enzyme
175 mixture (1 mg/ml collagenase/dispase, 0.05 mg/ml trypsin, Millipore Sigma) is added to dissociate
176 the tissues. Enzyme-inoculated cultures are incubated at room temperature for 1h with gentle
177 shaking. The dissociated tissues are centrifuged (800×g, 5 min, 4°C), and transferred to culture
178 containers as described.




179

180 **Figure 1.** Flow chart for establishment of honey bee-derived cell lines. HBSS, Hanks balanced
181 salt solution. See text for further details.

182 For smaller bee larvae (<4 mm), we mince the whole bodies immediately after sterilization. Eggs
183 are collected into 1.5 mL microfuge tubes containing medium and gently agitated so they remain in
184 suspension. They are sterilized and washed as above, then either minced with micro-scissors or
185 ground with a pestle. Cell cultures are maintained at 28 or 33°C and observed daily. Insect cell lines
186 are usually maintained at 28°C [61, 63-68]. We chose 33°C as a comparison temperature because the
187 honey bee brood nest temperature is maintained at 33-36°C for larval and pupal development [70].
188 Cultures are fed every 4 to 14 days (either by adding medium or replacing half, with these final
189 concentrations of antibiotics: 50 U/mL penicillin, 0.05 mg/mL streptomycin).
190 Over 600 honey bee cell cultures have been initiated using various combinations of tissues,
191 media and media additives (Table 3). An iterative process was conducted for developing cell lines,
192 i.e. we observed each culture initiated before deciding on the next media formulation to test.
193

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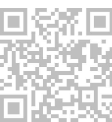
194 **Table 3.** Examples of basal media, nutrient supplements and media combinations tested in honey
195 bee cell culture initiations at BCIRL.

| **Basal medium**[1] | **Supplier** | **Results**[2] |
|---|---|---|
| EX-CELL 420 | Millipore Sigma, St Louis, MO | + |
| TNM-FH | Caisson | +/++ |
| Schneider's | Caisson | +/++ |
| L-15 | Caisson | - |
| IPL41 | Caisson | - |
| Shields and Sang | Caisson, Smithfield, UT | 0/+ |
| DMEM | Millipore Sigma | NT |
| RPMI-1640 | Millipore Sigma | NT |
| **Medium supplements** | | |
| 9% FBS (heat inactivated) | Millipore Sigma | +++ |
| 2% Insect medium supplement (IMS) | Millipore Sigma | -/0/+ |
| 1% MEM non-essential amino acids (NEA) | Millipore Sigma | -/0/+ |
| 10% Yeast extract | ThermoFisher Scientific, Waltham, MA | + |
| Royal jelly (RJ) | Made in-house[4] | ++/+++ |
| 10 µM 20-hydroxyecdysone | Cayman Chemical, Ann Arbor, MI | 0 |
| **Medium mixtures** | **Reference (if applicable)** | |
| HB-1 | [58] | +/++ |
| WH5 | [47] | + |
| Kimura's | [71] | + |
| EX-CELL 420 + L-15, 1:1 (CLG#2) | [67] | ++/+++ |
| TnMFH + IPL41, 1:1 (CLG#4) | N/A | + |
| Schneider's + TnMFH + L-15, 1:1:1 (CLG#5) | N/A | + |
| L-15 + EXCELL 420, 3:1 (HZ#1) | N/A | + |
| RPMI-1640 + EXCELL 420, 1:1 (HZ#2) | N/A | ++/+++ |
| DMEM+EXCELL 420, 1:1 (HZ#3) | N/A | -/0 |
| CLG#2 + RPMI1640 + DMEM, 2:1:1 (HZ#4) | N/A | -/0 |

196 [1]All basal media tested contained 9% FBS.
197 [2]Result key: [-], did not support cell health (vacuoles/granules/dark areas in the cytoplasm and/or
198 no cell attachment and/or cell lysis noted); [0], no visible impact; [+], initially encouraged cell
199 viability and attachment (≤1 month); [++], encouraged cell viability, attachment and replication for >
200 1 month; [+++], encouraged cell viability and replication such that the culture was passaged at least
201 1X. Combined scores indicate tissue dependent variability (e.g., -/+, [-] for eggs vs. [+] for queen
202 ovaries and midguts).
203 [3]NT = These basal media were only tested in combination with other media +/- supplements.
204 [4]Royal jelly was collected fresh from honey bee hives: 100 wax cells are washed off with 0.5 mL
205 CLG#2 and added to 100 mL CLG#2.

206 The influence of the media + FBS on overall cell health was evaluated by visual inspection before
207 testing different combinations of basal media or comparing the effects of supplements (nutritional or



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208 hormonal) added to the media. . For an example, we initiated cell lines with CLG#2, a combination
209 of an insect cell culture medium (EX-CELL 420) and a mammalian cell culture medium (L-15) used
210 to establish lepidopteran and hemipteran cell lines [67, 68]). The HZ media mixture series began with
211 the observation that CLG#2 produced healthy bee cell cultures. This was followed by testing
212 different ratios of the same basal media (HZ#1), which did not lead to cell replication. Next came the
213 replacement of one mammalian cell culture medium for another (RPMI-1640 for L-15, HZ#2), which
214 generated healthy cells similar to CLG#2. The next two media combinations (HZ#3 and #4) were
215 detrimental to cell viability. Similar iterations continued, each with a variety of media combinations
216 ± additives. In this process, we found that royal jelly positively influenced bee cell health, although
217 the underlying mechanisms for this improvement are not understand.

218 We pay particular attention to potential sources of contamination during cell line establishment.
219 Fungal contamination may occur in bee cell culture initiations, although in most cases, this is
220 controllable through surface sterilization and tissue washing as described. For tissues other than
221 neonates and eggs, a fungicide at low levels (e.g., 0.5 µg/mL amphotericin B) is initially incorporated
222 into culture media to minimize contamination. Another potential source of contamination is the
223 accidental inclusion of small hive beetle (*Aethina tumida*) tissues within primary cultures. Adult
224 beetles lay eggs in capped brood cells, as well as throughout the hive, and these eggs can be mistaken
225 for honey bee eggs [72]. *A. mellifera* only lay one egg per cell, while *A. tumida* can lay 10-30 eggs per
226 cell, with the beetle eggs about 2/3 the size of honey bee eggs. *A. tumida* larvae are smaller than honey
227 bee larvae, but they are more active, especially during their wandering stage
228 (https://beeaware.org.au/archive-pest/small-hive-beetle/#ad-image-0 [accessed 12/9/2019]). We take
229 care to ensure only honey bee eggs and larvae are collected when initiating primary tissue cultures.

230 The most promising and cleanest cultures were generated from eggs. Promising cultures consist
231 of viable-appearing, attached cells, with a clear cytoplasm, no vacuoles or darkened areas, and
232 distinct cell membranes. that are actively replicating (Fig. 2). Cultures in CLG#2 + FBS +/- royal jelly
233 led to the healthiest and longest enduring egg cell cultures. We have passaged eight egg cultures at
234 least once using 0.5% trypsin (3-5 min) and maintained the most promising cultures at 33$^{o}$C. HZ#2
235 medium also produced viable/replicating cell cultures, although none were passaged. These latter
236 cultures have a distinct major cell type different from cells in CLG#2 medium. Short-term egg cell
237 cultures (1 to 5 months) were initiated with TNM-FH and Schneider's + FBS.

238 Other short-term honey bee cell cultures (<1 month) that exhibit tissue and cell attachment, but
239 no or minimal cell replication, include those initiated from worker nervous system (in HB-1 or TNM-
240 FH + FBS), larval/worker/pupal midgut (in HB-1 or CLG#2 + FBS + YE), ground pupal whole head (in
241 CLG#2 + FBS), pupal nervous system (in HB-1), queen ovaries (using most basal media + FBS + other
242 supplements, and WH5 or Kimura's or HZ#1), queen midgut (in CLG#2 or TNM-FH or Kimura's +/-
243 other supplements) and queen/worker Malpighian tubules (in HZ#2 or Kimura's + FBS). Some
244 ovarian cell cultures exhibited cell networking with contractions. Based on these responses to
245 different media configurations, we propose that each tissue has its own nutrient/medium
246 requirements, which may reflect the *in vivo* situation. The tissues with the least stringent requirements
247 for generating short-term cultures, aside from egg cell cultures, are those from queen ovarian tissues.
248 This is generally true for cell lines initiated from ovaries and eggs, presumably due to the presence
249 of undifferentiated cells. Clearly more work is necessary to optimize the medium needed for each
250 tissue isolate.

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251

252 **Figure 2.** Representative images of attached, healthy cells from honey bee egg cell cultures in
253 CLG#2 + FBS, passaged one time, showing morphologically distinct cell types. E, elongated cells; S,
254 spherical cells; M, multi-sided cells; Ec, elongated cells growing out of a cell clump. Bars, 50 µm.

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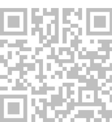
## 3. Cell lines for honey bee virus studies

Insect viruses typically infect cells derived from the host insect or from closely related species, with a few exceptions (e.g *Cricket paralysis virus*, which has an unusually wide host range). It follows therefore that honey bee viruses will replicate in honey bee-derived cell lines, and potentially in cell lines derived from other hymenopteran species (Table 2). The study of bee viruses in cell culture started with use of a primary cell line derived from the Asian honey bee (*Apis cerana*) [73]. SBV replicated in this primary cell line, and viral particles were seen by transmission electron microscopy (TEM) after 36 hours of infection. The establishment of a continuous honey bee cell line, AmE-711, was reported in 2013 [58] and was used in a single study of virus-virus interactions before the cell line crashed. Honey bees are typically infected by multiple viruses [74] and the AmE-711 cell line was used to examine *in vitro* competition between viruses in parallel with *in vivo* experiments [75]. Honey bee virus mixtures were fed to newly emerged honey bees, or used to infect AmE-711 cells with infection dynamics monitored by RT-qPCR [75]. Interestingly, IAPV had a higher replicative advantage among four different viruses (SBV, DWV, IAPV and BQCV) both *in vivo* and *in vitro* even when the virus mixture was predominantly composed of SBV. However, different infection dynamics were observed when KBV was present with a rapid increase in KBV rather than IAPV in cell culture. This work highlights the complexity of virus dynamics within a honey bee with the predominant virus determined in part by the composition of viruses within the honey bee virome at any given time. The results of these *in vitro* cell culture assays reflected virus dynamics observed on feeding of live bees, supporting the potential of a honey bee-derived cell line as a powerful tool to study virus infection dynamics.

Unfortunately, the AmE-711 cell line was persistently infected with DWV, as confirmed by sequence analysis and observation of DWV virions by TEM [75]. While the AmE-711 cell line could have been contaminated during- or subsequent to- establishment, the prevalence of DWV in honey bees and vertical transmission of this virus [76] suggest that DWV was present in the embryos that were used as starting material. Similarly, previously established primary cell lines as well as the genetically engineered continuous cell line MYN9 were also infected with DWV [47, 57]. As vertical transmission of DWV results from virus adherence to the surface of the egg (i.e. transovum transmission) [76], it should be possible to remove virus from the egg surface using a variety of published procedures [77]. In addition to providing a source of DWV virions, cell lines infected with DWV could be used to assess factors resulting in the switch from a covert to overt DWV infection. For the AmE-711 cell line, the suppressor of RNA interference from *Cricket paralysis virus*, CrPV-1A, was used to induce acute DWV infection and cytopathic effects, confirming RNAi-mediated suppression of DWV replication in these cells. The AmE-711 cell line was challenging to maintain, likely because environmental stressors (e.g. suboptimal medium, or environmental conditions) weakened the cells allowing DWV titers to increase, similar to the situation in honey bees [78, 79]. While the AmE-711 cell line crashed in 2015, it has since been recovered and still harbors DWV.

## 4. Establishment of virus-free cell lines

A variety of continuously replicating cell lines, including vertebrate and invertebrate lines, harbor viruses [80-83]. Next generation sequencing (NGS) facilitates the discovery of virus-derived sequences in cell lines, and has increased awareness of widespread covert infections in commonly used insect cell lines [84]. Given the widespread occurrence of virus-infected honey bee colonies [85],



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it is not surprising that virus contamination can be a major problem when establishing *A. mellifera* cell lines. One key example is the AmE-711 cell line, established from *A. mellifera* embryos, which is persistently infected with the DWV [75]. Two studies have described two different approaches for generating virus-free insect cell cultures.

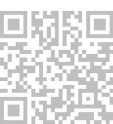
*4.1 Use of antiviral drugs to establish virus-free insect cell lines*

A nodavirus, named " Tn-nodavirus", was discovered in the BTI-TN-5B1-4 (Tn5) cell line derived from *Trichoplusia ni*, [86] and subsequently in a wide range of *T. ni* cell lines [80]. The IPLB-Sf21 cell line derived from *Spodoptera frugiperda* pupal ovaries, along with the subclonal line, Sf9, are well-recognized for generating recombinant proteins via the baculovirus expression system [87]. These Sf cell lines are infected with the Sf-rhabdovirus [81, 88]. Maghodia *et al.* (2017) first treated Sf9 cells with selected anti-viral agents, including ribavirin, 6-azauridune and/or vidarabine, for one month [80]. Although cultures with ribavirin initially appeared to be virus-free, they were later shown to contain virus when grown in medium without anti-viral drugs. The researchers then isolated single cells using limiting dilution and treated the subclones with antiviral agents. One virus-free clone was generated from this effort [80]. The Sf9-derived, virus-free Sf-RVN cell line is now commercially available (GlycoBac, Laramie, WY). The same drug-treatment procedure was repeated to remove the Tn-nodavirus from a *Trichoplusia ni* cell line (Tn-368) with similar results [80].

*4.2 Subcloning to establish a virus-free cell line*

Ma *et al*. (2019) used limiting dilution to generate virus-free Sf9 subclones in the absence of anti-viral agents from a mixed population of Sf9 cells comprised of two different virus variants (Sf-rhabdovirus $X^+$, $X^-$) and uninfected cells [81]. As individual cells failed to survive, a limiting dilution method was used to determine the minimum number of cells required for survival. They transferred 1000 cells/well into one column of a 96-well plate (final volume = 200µL) and made two-fold serial dilutions into subsequent wells. The wells containing the lowest cell numbers that reached more than 40% confluence after 6-8 weeks were transferred into 24-well plates. A total of 115 cell clones were obtained from fifteen 96-well plates and 18 of these tested as negative for Sf-rhabdovirus. Five of the 18 virus-free clones were further cultured for 30 passages and three of these clones were confirmed to be virus-free [81]. RNA-seq was used to confirm the absence of reads mapping to the Sf-rhabdovirus genome, for the virus-free cell clone, designated Sf-13F12.

While Sf9 and Tn-368 cells are rapidly replicating cell lines with doubling times of ~24 to 27 hr (https://web.expasy.org/cellosaurus), honey bee cell cultures to date have higher doubling times. The AmE-711 cell line for example was reported to double every 4 days [58]. This slow growth rate, combined with cells that are often difficult to culture, suggests that the limiting dilution method will be more challenging for bee cells. To promote cell replication, Reall *et al*. (2019) used conditioned medium from 72 hr old (log growth phase) parent cell lines, containing naturally produced growth factors, to generate clonal lines from *S. frugiperda* nervous system cell lines (7:3 conditioned medium to fresh medium)[89]. Cells were fed every 7 to 10 days with conditioned medium while in the 96-well plate and with fresh media after they were transferred into $T_{12.5}$ flasks. In ongoing research, we are using a similar procedure to isolate individual cell types from cell cultures that may contain both *A. tumida* and *A. mellifera* cells at BCIRL. Instead of using conditioned medium from potentially virus-



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containing parental lines, we generate conditioned medium from actively growing non-bee cell lines (free of bee viruses) and use it to supplement the fresh medium.

Maghodia *et al*. (2017) mentions additional methods that could be applied for cloning of *A. mellifera* cell lines [80], although many of these methods have not been attempted with insect cells. One classic method used to isolate insect cell subpopulations that could be applied to honey bee cells, involves soft agar/agarose overlays followed by colony picking. McIntosh and Rechtoris (1974) were the first to use this method on insect cell lines [90]. A more recent modification of this technique uses a feeder layer of actively replicating cells which is overlaid first with 0.2% ultra-pure agarose in 2X medium and then with 0.7% agarose in 2X medium. Low concentrations of well-dispersed cells are then mixed with 0.2% agarose in 2X medium + 72 hr conditioned medium (7:3, as above) to make the final layer [91]. In our hands, 0.5% agarose for the second layer led to better results with lepidopteran cells (Goodman, unpublished). Within a few weeks after the layers are set up, discrete colonies arising from single cells are removed with a pipette.

Based on the proven approaches described above, it should be feasible to establish virus-free honey bee-derived cell lines in the absence of DWV infection.

### *4.3 Potential use of CRISPR/Cas13 for establishing virus-free cell lines*

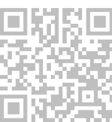
An emerging RNA targeting effector Cas13, an RNA-guided single stranded RNA ribonuclease [92], can be employed in conjunction with CRISPR to cleave single strand RNA including both mRNA and the single strand RNA genomes of some RNA viruses. The CRISPR/Cas13 tool has been applied for suppression of viral infections and for virus diagnosis [93]. For suppression of virus infection, CRISPR/Cas13 was transiently expressed in *Nicotiana benthamiana* leaves with guide RNAs (gRNA) targeting multiple regions of the small positive-strand RNA genome of *Turnip mosaic virus* (TuMV; Potyvirus). While gRNAs targeting different regions of the virus genome varied in efficiency, gRNAs targeting HC-pro and GFP sequences resulted in a >50% reduction in virus load [94]. As CRISPR/Cas9 tools have been widely applied in various insect cell lines [95, 96], it is conceivable that Cas13 could be employed for suppression of small RNA viruses such as DWV in honey bee-derived cell lines.

## 5. Potential applications of honey bee cell lines

The establishment of virus-free, honey bee cell lines will facilitate a number of avenues of research including 1) screening for antiviral compounds, 2) screening for the potential toxicity of insecticides to honey bees, 3) elucidation of honey bee-virus molecular interactions.

### *5.1 Screening of antiviral compounds for use in apiaries*

The cell culture system provides a powerful tool for high-throughput preliminary screening of antiviral drugs [97-99] prior to testing of candidate antiviral compounds in the whole organism. This cell line-based screening approach was used to identify candidate compounds for use against Zika virus [97, 98]. While the majority of screens have been conducted in mammalian cell lines, similar strategies could be employed in insect cell culture systems. For example, a high-throughput cell-based screening platform was established to mine compounds for lethality against mosquito cells (*Anopheles* and *Aedes*), but with little or no effect on other insect or human cell lines [100]. This screen resulted in identification of a mosquitocidal compound that had no effect on the vinegar fly, *Drosophila melanogaster*. A honey bee cell line could be employed 1) for screening of antiviral



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compounds to reduce viral load within a hive, 2) screening of current and candidate insecticides for safely to honey bees. The need for such a screening system was highlighted by the impact of neonicotinoid insecticides on honey bee populations [101, 102].

### *5.2 Elucidation of molecular virus - honey bee interactions*

A honey bee cell line would allow for in depth study of virus – host molecular interactions. This will be facilitated in particular by the establishment of infectious clones of honey bee viruses such as those of DWV [103, 104], that allow for reverse genetic analysis of gene function. Mechanisms of virus binding and entry into the cell, replication, encapsidation and release from the cell along with host cell antiviral response could be delineated by use of a honey bee cell line. A number of virus receptors have been identified from cell culture systems including those for Epstein-Barr virus (EBV) in human hematopoietic cells [105] and candidate dengue virus (DENV) receptors in mosquito cells [106]. Similarly, the DL2 and S2 cell lines derived from *D. melanogaster* have been used to study the infection cycle, replication of- and RNA interference associated with small RNA viruses that infect Drosophila [107-109].

Along with RNAi-, the emerging CRISPR/Cas9 gene editing tool, which has been used in several insect cell lines including Sf9, High Five, BmN [110], S2 [111, 112] and Aag2 [113], allows for identification of host genes involved in viral infection. For example, this system was used to confirm the role of the PIWI-interacting RNA (piRNA) pathway in antiviral response in mosquitoes [114]. A knockout mosquito cell line AF319 was generated by mutating *Dcr2*, a key gene in the RNA interference pathway, using the CRISPR/Cas9 technology. In the *Dcr2* knockout cell line, Piwi4 retained antiviral activity in the absence of the siRNA pathway [113]. The CRISPR/Cas9 gene editing tool also allows for functional characterization of genes on a genome-wide scale in cell culture systems, and has been used for the discovery of novel drug targets. For example, a CRISPR/Cas9 genome-wide gene knock-out assay in A549 cells was conducted to identify two host factors that are required for *Influenza A virus* (IAV) infection that could serve as targets for novel antiviral compounds [115]. Similar approaches to these could be adopted for identification of mechanisms of virus infection, and for antiviral targets for use in the protection of honey bees.

## **6. Conclusions**

Viruses play a significant role in honey bee losses. A honey bee cell line represents a valuable tool to identify solutions to virus infections in apiaries. Previous work with the AmE-711 cell line demonstrated the potential of honey bee cell lines to mirror *in vivo* virus dynamics. Cell lines derived from hymenopteran species other than *Apis mellifera* may support the replication of some viruses, but would be suboptimal for the study of honey bee-specific viruses.

Here we have summarized the establishment of primary and continuous cell lines derived from Hymenoptera. A systematic approach for establishment of cell lines with testing of multiple media is warranted for establishment of cell lines from less tractable species such as the honey bee. In addition, methods such as the use of antiviral drugs, sub-cloning and use of CRISPR/Cas13 could be employed for establishment of virus-free, honey bee cell lines. The use of a honey bee cell line in conjunction with virus replicons or infectious clones, and CRIPSR/Cas9-mediated genome editing will facilitate investigation of molecular virus-host interactions. Ultimately such studies will help mitigate virus-related honey bee losses.

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422

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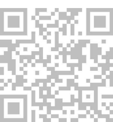

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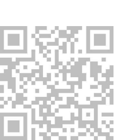

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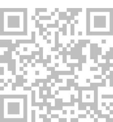

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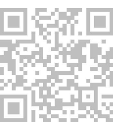

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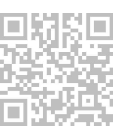

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