

1 **Vector-mediated viral transmission favours less virulent viruses**

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9

10 **Abstract**

11 While it is well-established that the ectoparasitic mite *Varroa destructor* is largely responsible
12 for the widely-reported decline of populations of the Western honeybee *Apis mellifera*, the
13 exact role the mite plays in honeybee health remains unclear. The last few years have seen a
14 surge in studies associating RNA viruses vectored by the mite with the death of honeybee
15 colonies. *Varroa* facilitates the spread of RNA viruses because it feeds on developing bee brood
16 and transfers haemolymph from bee-to-bee. Such a change in transmission, from horizontal and
17 vertical to vector-based, is predicted to lead to an increase in virulence of RNA viruses, thus
18 potentially providing an explanation for the observed association between *Varroa* and certain
19 viruses. Here we document the effect of changing the route of transmission of honeybee viruses
20 contained in the haemolymph of honeybee pupae. We find that a change in mode of
21 transmission rapidly increases viral titres of two honeybee viruses, Sacbrood virus (SBV) and
22 Black queen cell virus (BQCV). This increase in viral titre is accompanied by an increase in
23 virulence. In contrast, the virus most often associated with *Varroa*, Deformed wing virus
24 (DWV), shows a reduction in viral titre in the presence of SBV and BQCV. In addition, DWV
25 does not cause mortality to honeybee pupae in isolation. Most likely a change in mode of
26 transmission due to the arrival of a vector quickly eliminates the most virulent honeybee viruses
27 resulting in an association between *Varroa* and less virulent viruses such as DWV. Our work
28 therefore provides empirical evidence for an alternative explanation for the widely-observed
29 association between *Varroa* and DWV.

30

31 **Keywords:** Honeybee viruses, virulence, evolution, vector transmission.

32 **Introduction**

33 It is indisputable that the Western honeybee *Apis mellifera* suffers from the negative effects of
34 inappropriate use of pesticides¹ and a range of parasites and diseases². The most important
35 parasite today is the ectoparasitic mite *Varroa destructor*. The emergence of *V. destructor* is
36 the result of a host shift that occurred when *A. mellifera* and the Asian Hive Bee, *A. cerana*,
37 were brought into contact by beekeepers in the 1930s³.

38 *Varroa destructor* (hereafter simply referred to as *Varroa*) is aptly named. When left
39 untreated, *Varroa* typically destroys the colonies of its host⁴. In Europe and the United States
40 managed honeybee colonies suffer greatly from *Varroa* and require constant treatment with
41 miticides to prevent colonies from dying. At the same time, wild or feral honeybee populations
42 have been decimated or gone extinct⁵.

43 *Varroa* females feed on the haemolymph of the developing bees and in doing so are thought
44 to vector viruses carried therein^{6,7}. Although a variety of viruses could potentially be
45 transmitted by *Varroa*⁸, one in particular – Deformed wing virus (DWV) – is strongly
46 associated with *Varroa*. For example, as *Varroa* sequentially invaded the islands of Hawaii,
47 viral titres of DWV increased, while the diversity of DWV viral strains decreased, such that a
48 single strain came to dominate after a few years⁹. A similar phenomenon was seen in New
49 Zealand where titres of DWV dramatically increased with the length of exposure to *Varroa*¹⁰.

50 Vector-based transmission is predicted to lead to an increase in virulence because it changes
51 the evolutionary trade-off between virulence and transmission¹¹. While an obligate parasite is
52 selected to replicate quickly, so that it can infect as many hosts as possible, a high rate of
53 replication may kill the host before the parasite is transmitted to its next host. Selection will
54 thus act against a pathogen that kills or immobilises its host if this reduces its long-term
55 transmission success^{12,13}. The arrival of a vector changes the dynamics of the transmission-
56 virulence trade-off. If a pathogen can harness a mobile vector to facilitate its spread to new
57 hosts, then it no longer relies on its current host for transmission.

58 An increase in virulence after a change in route of transmission was recently documented in
59 the obligate endosymbiont *Wolbachia* and one of its native hosts, the isopod *Armadillidium*
60 *vulgare*. Because *Wolbachia* is normally transmitted vertically, via eggs, it requires its host to
61 be alive and reach reproductive age. Hence, *Wolbachia* tends to form symbiotic relationships
62 with its hosts. However, when the route of transmission was changed from vertical to
63 horizontal, by injecting *Wolbachia* directly into the haemolymph of the host, *Wolbachia* titres
64 quickly escalated and infections became highly virulent, resulting in the death of the hosts after
65 only a few serial passages¹⁴.

66 At first glance the association between *Varroa* and DWV seems to fit the predicted change in
67 virulence after the arrival of a vector, and thus a change in mode of transmission. However,
68 honey bees host many viruses that are both common and widespread^{8,15} including viruses that,
69 like DWV, are present in *Varroa* and can also be vector-transmitted (eg. viruses of the Acute
70 bee paralysis virus complex (ABPV and Kashmir bee virus, etc.)¹⁶). This raises the question:
71 why has DWV become synonymous with *Varroa* infestation, but not other honey bee viruses?
72 An alternative explanation for the observed association is that more virulent viruses are
73 eliminated from the population due to excessive host mortality following vector-based
74 transmission, thereby allowing less virulent DWV to take the upper hand^{10,17,18}. Here we test
75 this alternative explanation empirically using a population of honeybees naïve to both *Varroa*
76 and DWV.

77 We experimentally changed the transmission of bee viruses from horizontal (via faeces and
78 feeding) to vector-mediated transmission by performing a serial passage experiment. We
79 injected extracts from bee pupa to bee pupa repeatedly for up to 30 transmission cycles and
80 found that two viruses naturally present in our bee population, Sacbrood virus (SBV) and Black
81 queen cell virus (BQCV), rapidly increased in titre. In contrast, DWV introduced via injection
82 rapidly decreased in titre accompanied by a rapid increase in titres of SBV and BQCV. More
83 importantly, DWV alone did not cause mortality in pupae, whereas injection with serially
84 passaged bee extracts containing high titres of SBV and BQCV did. We conclude that the
85 observed association between *Varroa* and DWV may not necessarily be due to *Varroa*
86 increasing the virulence of DWV, but could be explained by *Varroa* eliminating other viruses
87 that become more virulent when the mode of transmission changes.

88

89 **Results**

90 *Experimental overview*

91 To mimic the effects of changing to a new, vector-based transmission route we serially injected
92 honey bee pupae with viruses and monitored the changes in virus levels. Injecting honeybee
93 extracts into pupae has previously been used to incubate viruses prior to serological
94 experiments¹⁹ and to obtain standardised inoculum for injection experiments²⁰. We adapted this
95 protocol to conduct serial transmission of honeybee extracts by pupal injection for 20+
96 transmission cycles. We performed two independent transmission experiments with different
97 starting inoculum: (1) extracts obtained from asymptomatic (DWV-naïve) honeybees; and (2)
98 extracts obtained from symptomatic (DWV-infected) honeybees.

99

100 *1. Serial transmission of asymptomatic (DWV-naïve) inoculum*

101 In our first experiment (Figure 1A; Serial Transmission 1), we took our starting inoculum from
102 adults sampled from three asymptomatic honeybee colonies from Sydney, Australia (lacking
103 DWV and naïve to *Varroa*, referred to hereafter as colonies 1, 2 and 3). We subjected white-
104 eyed pupae from the same three colonies to each of three treatments: (1) pupae injected with
105 inoculum containing viruses; (2) pupae injected with extraction buffer as a procedural control
106 ('buffer'); and (3) pupae left unmanipulated ('control'). After 4 days, we harvested pupae for
107 extraction to generate inoculum for the next transmission cycle. We passaged inoculum for 20
108 transmission cycles (18 for colony 3; see Materials and Methods).

109 We used end-point PCR to screen for the presence of the five known viruses present in
110 Australia²¹ in our initial adult workers and in pupae sampled at regular intervals during the 18-
111 20 serial transmission cycles. We detected two just two viruses: Sacbrood virus (SBV) and
112 Black Queen Cell virus (BQCV). Control pupae did not test positive for SBV and BQCV. In
113 contrast, buffer-injected procedural controls occasionally tested positive for SBV and BQCV.
114 It has been well documented that the effect of injection procedure alone can cause the irruption
115 of latent viral diseases in bees¹⁹, in line with our observations of SBV and BQCV in our buffer,
116 but not unmanipulated, control pupae.

117

118 *Serial transmission results in a rapid increase in viral titre*

119 To determine whether serial transmission resulted in increased viral titres, we assessed
120 expression levels of SBV and BQCV using quantitative PCR and compared those to the
121 expression levels of two endogenous control genes, *Actin* and *Rps5* (see Materials and
122 Methods). We standardised between the three independent colonies and transmission cycles by
123 re-injecting bee extract from colonies 1-3, transmission cycles 1, 5, 7, 10, 15 and 18 (colony 3)

124 or 20 (colonies 1 and 2) into pupae sourced from an independent colony and performed qPCR
125 on these samples, together with buffer-injected and unmanipulated controls.

126 Both SBV and BQCV virus showed a rapid increase in titre (Figure 2, Table S2). Compared
127 to control and buffer-injected pupae, BQCV levels increased in pupae injected with bee extract
128 after only one transmission cycle, after which levels remained the same (Figure 2 A). Levels of
129 SBV remained low after one transmission cycle but had increased by transmission cycle 5 and
130 remained high thereafter (Figure 2 B).

131 To correlate viral titres as measured by qPCR to total RNA content, we examined the amount
132 of viral RNA in pupae injected with bee extract after 20 transmission cycles (colonies 1 and 2,
133 as colony 3 was no longer available due to the loss of the colony's queen) using HiSeq
134 (Illumina) total RNA sequencing. BQCV and SBV levels made up the vast majority of non-
135 ribosomal RNA in pupae, collectively accounting for 92.6% and 86% of total RNA in colony
136 1 and 2 pupae, respectively. BQCV levels reached approximately 60%, while SBV levels
137 ranged between 26-35% (Figure 3 A, Table S3).

138

139 *2. Serial transmission of DWV results in a decrease in DWV titre*

140 We then repeated our serial transmission experiment (Figure 1A, Serial Transmission 2) using
141 inoculum obtained from 5 symptomatic, DWV-infected adult bees from New Zealand (see
142 Material and Methods, including details of quarantine permits), and injecting into lab reared
143 pupae obtained from 2 independent recipient honeybee colonies (referred to as colony 4 and 5;
144 naïve to both DWV and *Varroa*). We passaged inoculum for 30 transmission cycles. We
145 quantified the total amount of viral RNA in the initial adults (DWV source) and after 1, 10, 20
146 and 30 transmission cycles in pupae 4 days post-injection, along with buffer-injected and
147 control pupae taken from cycle 20 using HiSeq (Illumina) sequencing (Figure 3 B, Table S3).

148 Almost 90% of non-ribosomal RNA came from DWV in our original inoculum, suggesting
149 that the viral load of symptomatic honey bees can reach extreme levels (Figure 3 B; 'DWV
150 source'; Table S3). After one transmission cycle, DWV levels reached 25-32% of total non-
151 ribosomal RNA in injected pupae from both colonies. Thereafter, DWV levels decreased
152 rapidly until only a small amount (<10%) of RNA could be attributed to DWV after 10
153 transmission cycles (Figure 3 B; Table S3). The decrease in DWV titres was accompanied by
154 an increase in BQCV and SBV titre (Figure 3 B), similar to the increase seen in our serial
155 transmission experiment without the inclusion of DWV (Figure 3 A). In the buffer injected
156 pupae from colony 4, we also saw high levels of SBV, indicating that the injection procedure
157 alone can result in increase in endogenous virus levels, in line with previous observations¹⁹.

158 We also saw a shift in DWV strain composition. DWV is known to comprise of 3 main master
159 variants: strain DWV-A, DWV-B and DWV-C^{22,23}. Strain A is globally associated with
160 increased viral titres and colony decline^{9,24}. Strain B is an emerging DWV genotype that has
161 increased virulence compared to DWV-A in laboratory experiments, but has also been found
162 in colonies that seem to cope with the presence of *Varroa*^{17,20,22,23} (the effect of strain C is
163 currently unknown). Our original inoculum contained low amounts of strain B (0.34% of total
164 viral RNA) which had increased after 20 transmission cycles, particularly in colony 5 (1.66%),
165 only to drop again after 30 cycles (Figure 3 B, Table S3). The total amount of RNA attributable
166 to virus ranged between 88-97% in pupae injected with virus inoculum at all cycles tested, in
167 contrast with control (0.3-0.4% virus) and buffer samples (62% in colony 4 (mentioned above),
168 and 0.18% in colony 5).

169

170 *Injecting pupae with serially transmitted SBV and BQCV results in high mortality while DWV*
171 *alone does not*

172 To compare the virulence of our serially passaged extracts, we injected lab-reared white-eyed
173 pupae with inoculum extracted from our DWV source adults, inoculum from Serial
174 transmission experiment 1, cycle 20 (containing BQCV/SBV, without DWV), and inoculum
175 from Serial transmission experiment 2, cycle 20 (containing DWV/BQCV/SBV). We
176 performed two independent mortality experiments, testing inoculum from cycle 20 from colony
177 1 and 4 in one independent source colony, and colony 2 and 5 in a second independent source
178 colony (see Figure 1 B for schematic).

179 Overall survival was significantly affected by treatment in both assays (respectively $\chi^2_4 =$
180 235.68, $p < 0.00001$, $n = 300$ and $\chi^2_4 = 355.21$, $p < 0.00001$, $n = 300$; Table S4). Mortality of
181 pupae when injected with DWV alone was not statistically different from buffer-injected
182 controls (both $p > 0.153$; Figure 4, Table S4). When pupae were injected with cycle 20 inoculum
183 from both serial transmission experiments, mortality between inoculum with and without DWV
184 were not statistically different (both $p > 0.068$; Figure 4, Table S4). In both instances, mortality
185 was much higher compared to buffer-injected pupae and pupae injected with DWV alone (all
186 $p < 0.00001$; Figure 4, Table S4). Clearly, increased mortality is due to the increased titres of
187 SBV and BQCV, not due to the presence of DWV. When testing the effect of ‘source colony’
188 on pupae survival we found that our first source colony had a significantly higher survival than
189 the second ($\chi^2_1 = 4.90$, $p = 0.0268$, $n = 600$). However, the overall result was the same for both
190 colonies. ‘Replicate’ had no significant effect on survival in both colonies (respectively $\chi^2_4 =$
191 5.87, $p = 0.209$, $n = 300$ and $\chi^2_4 = 8.84$, $p = 0.065$, $n = 300$, Table S4).

192

193 **Discussion**

194 We aimed to investigate the effect of changing the route of transmission, from horizontal and,
195 in some cases vertical, to vector-transmitted, to determine if such a change in route of
196 transmission alone is sufficient to increase virulence of RNA viruses contained in the
197 haemolymph of honeybees. We found that two viruses, Sacbrood virus (SBV) and Black queen
198 cell virus (BQCV) rapidly increased in titres when injected into white-eyed pupae. In contrast,
199 when we injected inoculum containing high titres of Deformed wing virus (DWV) strain A,
200 DWV viral titres rapidly decreased, most likely due to competition with SBV and BQCV.
201 Interestingly, injecting high titres of DWV strain A into pupae did not result in the death of the
202 pupae, indicating that this strain of DWV does not kill developing brood. Injecting high titres
203 of SBV and BQCV did result in high mortality.

204 Both SBV and BQCV are brood diseases; young larvae normally become infected early on
205 via feeding by adult bees²⁵. When brood dies from either virus, nurse bees will remove and
206 partially cannibalise the dead brood, thus themselves accumulating the virus. Because both
207 viruses end up in the bees' hypopharyngeal gland (in which brood food is produced), nurse bees
208 transmit the viruses when feeding young larvae²⁵. Under natural conditions, and in the absence
209 of *Varroa*, both SBV and BQCV were found to occur at a frequency of around 10% in summer
210 in Britain using immunodiffusion tests²⁶. Both viruses are easily detected when bee extract from
211 adult bees is injected into pupae²⁷, indicating that both viruses are present at low incidences
212 without causing overt infections, and readily amplify upon injection into pupae. In Australia,
213 BQCV was found in 65% and SBV in 35% of hives using more sensitive molecular detection
214 methods, further indicating high viral prevalence in the absence of overt infections²¹. Our
215 results suggest that repeated vector-mediated transmission of bee extract containing SBV or
216 BQCV will rapidly lead to such high viral titres that the brood never develops to adulthood.
217 Our experimental conditions were restricted to pupae, as our quarantine permits required
218 injected pupae to be terminated prior to eclosion. Therefore, our results reflect conditions that
219 are favourable to replication in brood, as we harvested injected pupae randomly, regardless of
220 whether they would have successfully eclosed. Considering that *Varroa* parasitises brood
221 initially, the process of vector-mediated transmission similarly begins in brood. However in
222 contrast to our experimental conditions, only those surviving to eclosion will harbour the
223 viruses that are selected for. This suggests that *Varroa* selects against high replication of viruses
224 causing brood mortality, whereas our selective regime did the opposite.

225 Another virus commonly found in honey bees, Acute Bee Paralysis Virus (ABPV), cannot
226 replicate when injected into pupae that already contain either SBV or BQCV²⁶, showing that
227 indeed SBV and BQCV are highly competitive, probably due to their ability to replicate rapidly.

228 ABPV, and the closely related Kashmir Bee virus (KBV) and Israeli Acute Bee virus (IAPV),
229 are often the first viruses to be associated with the arrival of *Varroa* before they are gradually
230 displaced by DWV^{9,28}. A study documenting the change in viral landscape as *Varroa* invaded
231 the islands of New Zealand, found negative associations between KBV and DWV and between
232 DWV and SBV in both bee and mite samples, while SBV and BQCV were positively associated
233 in both bees and mites¹⁰. As the time since the arrival of *Varroa* increased, the prevalence of
234 KBV, SBV and BQCV decreased, while DWV increased¹⁰. These results are consistent with
235 the hypothesis that the succession of honeybee viruses after the arrival of *Varroa* is due to the
236 most virulent viruses being selected against, if *Varroa* transmission facilitates an increase in
237 replication rate^{6,18}. Our results are the first to provide experimental evidence for this
238 hypothesis.

239 Clearly honeybee colonies contain a number of different viruses, both of different species as
240 well as different strains of the same species given the high mutation and replication rates of
241 RNA viruses²⁹. Competition amongst viruses drives virulence^{30,31}. Inevitably some viruses are
242 more virulent than others. When virulence is too high, the host is likely to die before it has a
243 chance to emerge and transmit the virus to other bees via feeding or faeces. Thus, viruses that
244 are too virulent will be selected against. The arrival of a vector changes the dynamics, as now
245 even highly virulent strains can be transmitted if they manage to get into the vector. But such
246 an increase in the prevalence of highly virulent viruses is bound to be temporary if the vector
247 is killed in the process. Because a vector such as *Varroa* depends on the bee to complete its
248 development (the female mites emerge from the brood cell together with the emerging bee), its
249 arrival will not improve long-term transmissibility of virulent variants, thus leading to the
250 succession from highly virulent viral species to less virulent species as documented in New
251 Zealand¹⁰.

252 The last few years have seen a surge in publications that link the arrival of *Varroa* to the
253 emergence of specific strains of DWV^{9,10,22-24}. Initially it was thought that DWV strain A was
254 the most virulent strain while strain B was considered to be more benign^{22,32}. However, this
255 simple interpretation now seems questionable, as recently strain B has been associated with
256 colony losses¹⁷ and appears to be more virulent in an experimental setting²⁰. Regardless, the
257 prevailing wisdom is that *Varroa* has led to a change in virulence of an otherwise relatively
258 benign virus by changing the virus' mode of transmission, thus modifying the virulence-
259 transmission tradeoff⁹. The association between DWV and *Varroa* is so strong, that many now
260 claim that it is the virus that needs to be controlled, not the mite, if we want to protect the bees.
261 We offer experimental evidence for an alternative explanation for the association between
262 *Varroa* and DWV. In the presence of more virulent viruses such as SBV and BQCV, DWV is

263 outcompeted and, if present at all, often below detection level in the absence of *Varroa*. The
264 arrival of *Varroa* quickly selects for an increase in the prevalence of the most virulent viruses
265 until they become so virulent their transmission grinds to a halt due to the death of the brood
266 and thus the mites. Now more benign viruses such as DWV can make their appearance. Hence,
267 perhaps instead of *Varroa* actively selecting for specific, virulent strains of DWV, DWV is
268 simply ‘the last virus standing’ after more virulent species have been selected against.

269

270 **Materials and Methods**

271 We used honeybees (*Apis mellifera*) of standard Australian commercial stock that had been
272 kept at the University of Sydney’s apiary for multiple years without showing any symptoms of
273 disease. *Varroa* is not present in Australia. Moreover, it is widely accepted that DWV is not
274 established in Australia after a recent comprehensive, country-wide survey²¹. A second study
275 showed that strains distantly related to DWV are present in the northern states of Australia (NT
276 and QLD). Next-generation transcriptome sequencing identified contigs showing 53-69%
277 amino acid identity to DWV strains A and B³³. This indicates that in some regions of Australia,
278 honeybees host a related virus that is distinct from previously characterised DWV variants.
279 Nevertheless, the colonies used in all of our experiments were sourced from regions where no
280 trace of DWV has been identified in previous surveys, including our own. In addition, we did
281 not detect DWV in our serial transmission experiment or in subsequent next generation
282 sequencing of controls (see further). We thus conclude that our bees were naïve to DWV.

283

284 *1. Serial transmission experiment*

285 *1.1 Inoculum preparation*

286 We modified the extraction protocol from Roberts and Anderson³⁴. For our first serial
287 transmission experiment, we sampled healthy, DWV-naïve adult bees collected from hive
288 entrances of three separate colonies (colonies 1, 2 and 3, Figure 1A). For each colony, we
289 crushed the thorax and abdomen of five bees in 2ml 0.5 M potassium phosphate buffer (pH 8),
290 removed the lysate by pipetting, then added 5% v/v diethyl ether and 10% v/v chloroform and
291 centrifuged the tubes at 12,000 rpm for 2 minutes. We removed the supernatant and filtered it
292 through a 0.22 µm bacterial filter to remove non-viral pathogens. We then diluted the extracts
293 with potassium phosphate buffer by a factor of 10⁻³. Dilution was necessary because injection
294 of undiluted honeybee extract rapidly kills pupae, potentially due to carryover of toxic
295 metabolites (J. Roberts; personal communication). This dilution factor was chosen based on
296 pilot experiments, where we injected 10-fold serial dilutions of adult and pupal bee extracts into
297 white-eye pupae (ranging from undiluted through to 10⁻⁶). A 10⁻³ dilution gave the highest

298 concentration that showed no signs of lethality 1 day post-injection. We added 10% v/v green
299 food dye to the bee extract prior to injection to check if injections had been successful. We
300 injected the DWV-free inoculum obtained from each colony into pupae obtained from the same
301 three colonies (1, 2 and 3) as described below.

302 To obtain DWV for our second serial transmission experiment, we sourced bees visibly
303 showing symptoms of DWV from the top bars of frames from *Varroa* infected colonies in New
304 Zealand (see below for Quarantine details). We cut the thorax and abdomen of five adult bees
305 sagittally in halves and used one half of each bee for inoculum preparation as described above.
306 We kept the other half at -70°C under quarantine conditions for later whole-transcriptome
307 sequencing. We used DWV-containing inoculum as the starting material for injecting into three
308 independent colonies (colonies 4, 5 and 6) as described below.

309

310 *1.2 Injection Procedure*

311 We took 75 white-eyed pupae from their brood comb from each of the six experimental
312 colonies, and distributed pupae into three treatment groups of 25: experimental, buffer and
313 control. We injected the experimental group with 2 µL of initial honeybee inoculum using a
314 Hamilton 10 µl syringe and a 0.3mm needle. As described above, pupae from colonies 1, 2 and
315 3 were injected with 2µl inoculum taken from DWV-free, asymptomatic nestmate bees, and
316 pupae from colonies 4, 5 and 6 were injected with inoculum obtained from DWV-symptomatic
317 bees from New Zealand (Figure 1 A). We injected the buffer group (procedural control) with 2
318 µL of potassium phosphate buffer to control for the effect of injection. Experimental and buffer
319 group pupae were injected between the fourth and fifth abdominal tergites. We did not perform
320 any further manipulations on the control group. After injections we placed pupae in petri dishes
321 lined with filter paper soaked in 12% glycerol and incubated them at 34.5°C. All pupae were
322 stored in the lab in our approved quarantine facility under quarantine conditions. After 4 days,
323 we froze the pupae at -70°C until required. To prepare for the next round of injections we
324 selected five pupae for extraction using a random number generator. We randomly selected
325 another five to determine viral levels using real-time quantitative PCR, and kept the remaining
326 fifteen in reserve. Extracts from previous transmission cycles were then injected into the next
327 round of white-eyed pupae collected from brood combs originating from the same six
328 experimental colonies. For the DWV-naïve transmission experiment, we concluded a total of
329 20 transmission cycles for colonies 1 and 2 whereas the third colony replaced its queen so that
330 we were unable to collect pupae beyond 18 transmission cycles. For our DWV-positive
331 transmission experiment, we concluded 30 transmission cycles for colonies 4 and 5, whereas

332 the queen from colony 6 was replaced during the 4th transmission cycle and thus this colony
333 was excluded from any further analysis.

334

335 *1.3 Detection of viruses*

336 We used end-point PCR to screen for the presence of viruses in our starting colonies. We used
337 Trizol (Life Technologies) to extract RNA from 12 uninjected bees collected from colonies 1-
338 3 at the beginning of the first serial transmission experiment. For our second serial transmission
339 experiment we extracted RNA from 6 pupae from colonies 4-6, sampled at the time of
340 transmission cycle 1. We quantified RNA using a Qubit Broad Range Assay (Life
341 Technologies), and normalised to 200 ng/ μ l before treatment with DNase. We synthesised first
342 strand cDNA from 0.5 μ g total RNA using SuperScript III Reverse Transcriptase (Invitrogen)
343 and random hexamer primers. We performed PCRs to screen for presence of BQCV, SBV,
344 IAPV and Lake Sinai Virus (LSV), as these viruses are most commonly found in Australian
345 bees ²¹, using the primers described in Table S1 with an initial 5 min denaturing step at 94 °C,
346 followed by 38 cycles of 94 °C for 1 min, annealing temperature for 1 min and 72 °C for 1 min
347 per kb of product, with a final extension step for 10 min at 72 °C. We visualised PCR products
348 on a 1.5% agarose gel using SYBR Safe DNA stain (Life Technologies). Positive PCR products
349 were sequenced by Macrogen and identity confirmed by BLAST to NCBI GenBank online
350 database.

351 We screened for viruses throughout the experiment using endpoint PCR at various timepoints.
352 For serial transmission experiment 1, we examined 5 pupae from all three treatment groups at
353 transmission cycles 1, 5, 7, 10, 13 and 15. For serial transmission experiment 2 we examined 3
354 pupae from all treatment groups at transmission cycles 1, 10, and 20 to validate the presence of
355 DWV in our experimental group, and the absence of DWV from our buffer and control groups.
356

357 *2. Assessment of virus levels*

358 *2.1 Serial transmission experiment 1- Quantitative Real-time PCR*

359 To compare the viral titres between colonies 1-3 after serial transmission with extracts sourced
360 from DWV-naïve bees, we collected white-eyed pupae from an independent colony to
361 standardise for colony background. We injected bee extracts from experimental groups of
362 colonies 1-3, from transmission cycles 1, 5, 7, 10, 15 and 20 (cycle 18 for colony 3), into 10
363 white-eyed pupae, along with 10 buffer-injected procedural controls and 10 uninjected
364 (unmanipulated) controls (see Figure 1 B for a schematic representation of the experiment). We
365 randomly selected five pupae from each group and extracted RNA from each pupa separately
366 in 1 mL of Trizol. We treated the RNA with DNase and performed cDNA synthesis using the

367 same method described above. We then diluted cDNA to a final concentration of 27 ng/μl. We
368 created negative controls for the qPCR assay by pooling extracted RNA from samples drawn
369 from the same treatment group and transmission cycle, treating them with DNase and mixing
370 them with all the reagents for cDNA synthesis except the reverse transcriptase enzyme. We
371 created the standards for our qPCR assay by taking previous cDNA samples with high levels
372 of SBV and BQCV, then performing a serial dilution over 3 orders of magnitude.

373 We designed qPCR primers to amplify SBV and BQCV and used previously published
374 primers for β-Actin, Efl-α, and Rps5, which served as endogenous controls (Table S1). We
375 confirmed the specificity of each primer pair via melt-curve analysis and gel electrophoresis.
376 We performed the assay using a Roche LightCycler 480 using 2x SYBR Master Mix (Roche
377 Technologies). We pre-incubated the reactions (95°C, 10 minutes) prior to 45 amplification
378 cycles (95°C, 10 seconds; 58°C 10 seconds; 72°C, 10 seconds), and measured fluorescence at
379 each extension step. We obtained Cq values using the second derivative maximum method
380 using the Roche LightCycler 480 software. The same software was used to calculate the
381 efficiencies of each set of primers from the standard curves on each plate.

382

383 *Statistical analysis – quantitative real-time PCR*

384 We compared the stability of each reference gene in Bestkeeper³⁵ and used the two most stable
385 reference genes, *Actin* and *Rps5*, to normalise the expression of BQCV and SBV in all samples.
386 The expression level of each gene was calculated as $E^{Cq_{min} - Cq_i}$, where E is the efficiency of the
387 primers calculated, Cq_{min} is the lowest Cq value for a given gene and Cq_i is the Cq of sample *i*.
388 The expression of each of SBV and BQCV in each sample was then normalised against the
389 geometric mean of the expression levels for both reference genes to obtain the final relative
390 expression score³⁶. To compare the viral titres between each transmission cycle and between
391 colonies 1-3 in our pupal quantitative PCR, we performed a one-way ANOVA (Table S2).

392

393 *2.2 Serial transmission experiment 2- Whole transcriptome RNA sequencing*

394 To compare virus levels between colonies 4 and 5 after serial transmission with DWV-
395 inoculum, we extracted RNA from five randomly selected pupae 4 days post-injection for each
396 of transmission cycles 1, 10, 20 and 30, as well as buffer injected and unmanipulated control
397 pupae from transmission cycle 20. We also extracted RNA from five pupae from transmission
398 cycle 20 from colonies 1 and 2 from serial transmission experiment 1 (DWV-naïve
399 transmission). Finally, we extracted RNA from our DWV source population, using the stored
400 half of adult bees from which the haemolymph containing DWV was initially extracted (see
401 Figure 1 B for schematic). We extracted RNA using 1mL Trizol as outlined above. After DNase

402 treatment, we used an RNeasy Mini Kit (Qiagen) to clean RNA to a total of $\geq 5\mu\text{g}$. Cleaned
403 RNA was diluted to a minimum of $100\text{ng}/\mu\text{L}$ into a $50\mu\text{L}$ aliquot and stored at -70°C until it
404 was shipped on dry ice to the Australian Genome Research Facility (AGRF) laboratory
405 (Melbourne, Australia) for preparation of whole transcriptome, 100bp paired-end library with
406 ribosome depletion (Ribo-zero Gold (human/mouse/rat)), and HiSeq (Illumina) sequencing.
407 We multiplexed fifteen libraries across two lanes, providing between 3.13-4.2 Gb data per
408 library, for a total data yield of 56.74 Gb. The raw sequencing reads from this project have been
409 deposited to Genbank under the Bioproject ID PRJNA397460 at the Sequence Read Archive
410 (SRA Study ID: SRP114989).

411

412 *Sequencing - data analysis*

413 We performed an initial *de novo* assembly of sequencing reads for each sample using Trinity³⁷.
414 To determine which honey bee viruses were present, we used BLAST searches to compare
415 Trinity-assembled contigs to a custom honey bee virus database containing all currently known
416 honey bee virus genome sequences. We found contigs matching to BQCV and SBV in all
417 samples. DWV contigs were present in assemblies from the DWV source population and cycles
418 1-30 of the DWV serial transmission experiment. In addition, the DWV source population also
419 contained contigs matching to the recently described Apis Rhabdovirus 1 and 2 (ARV-1 and
420 ARV-2)³⁸. However the levels of these viruses were below 0.05% of the total RNA reads (Table
421 S3), and were subsequently not detected in any further transmission cycles. We found no other
422 viruses in our samples. Interestingly, short contigs for DWV were also assembled from our
423 buffer injected and unmanipulated controls. Previous studies using Illumina HiSeq and MiSeq
424 technology have reported 'sample bleeding' due to reads being incorrectly assigned to the wrong
425 sample source when multiplexed in the same sequencing lane^{20,39}. To assess the level of DWV
426 read misassignment in our buffer and control samples, we aligned sequencing reads of each
427 sample to DWV-A and DWV-B genomes using Bowtie2⁴⁰. All control samples showed less
428 than 0.02% of total reads aligning to DWV. This level was similar to the level of multiplex
429 sample bleeding that we observed in a negative control sample. In addition, we were unable to
430 amplify DWV using PCR from cDNA synthesised from our buffer and control samples,
431 therefore we concluded that the DWV contigs present in our controls are a result of inaccurate
432 sample assignment of reads during the multiplexed HiSeq sequencing run.

433 Prior to analysing the viral content of our transcriptomes, we assessed the proportion of
434 residual ribosomal RNA (rRNA) reads, as complete ribosome depletion may not be obtained
435 when using Ribo Zero Gold (human/mouse/rat) for invertebrate samples. We identified *Apis*
436 *mellifera* rRNA from Trinity contigs by BLAST searches and aligned reads for each sample

437 using Bowtie2. The percentage of residual rRNA reads ranged from 2.5 - 46% of total RNA
438 reads per library (Table S3). These values were factored into any subsequent viral percentage
439 calculations.

440 To determine the viral content in each treatment condition, we aligned sequencing reads of
441 each sample to BQCV, SBV, DWV-A and DWV-B genomes using Bowtie2. We used
442 representative SBV and BQCV contigs assembled *de novo* from our samples as the template
443 for Bowtie2 alignments, as our SBV and BQCV strains differed significantly to the reference
444 SBV and BQCV genomes from Genbank (SBV: 92.6% nucleotide identity to AF092924⁴¹;
445 BQCV: 89.2% nucleotide identity to AF183905⁴²). We used DWV-A and B reference
446 sequences available in Genbank (AJ489744 and AY251269) as the template for Bowtie2
447 alignments, as the DWV strain assembled from our DWV source population matched to DWV-
448 A with high nucleotide identity (98.7%). The reference SBV, BQCV and source DWV
449 sequences used in this study have been deposited to Genbank under accession numbers
450 MF623170, MF623171 and MF623172. We compared the SBV and BQCV sequences from our
451 study to published available SBV and BQCV genomes. We performed nucleotide alignments
452 in Geneious (v10.2.4⁴³) using Muscle and generated phylogenetic trees using PhyML (Figure
453 S1).

454

455 3. Assessment of virulence

456 3.1 Pupal survival screen

457 To assess the virulence of the viruses contained in the inoculum generated after serial
458 transmission cycles in experiment 1 (containing SBV/BQCV) and experiment 2 (containing
459 DWV/SBV/BQCV), we developed a mortality assay using change in pupal eye colour to
460 determine pupal mortality. As pupae develop, pigments such as ommochromin are deposited in
461 the compound eyes and ocelli, causing a change in colour from white, through pink and red, to
462 the endpoint black⁴⁴ (see Figure S2). By comparing the colour of a pupa's eyes over consecutive
463 days, we developed an assay that allowed us to determine the point in time a pupa died. A pupa
464 was determined to have died when its eyes had ceased changing colour over two consecutive
465 photographs, and/or when the compound eye had retracted from the cuticle (Figure S2).

466 We sourced pupae from 2 independent honeybee colonies and injected them with inoculum
467 as per our serial transmission protocol. We injected inoculum from transmission cycle 20 from
468 Colony 1 (BQCV/SBV) and Colony 4 (DWV/BQCV/SBV) for the first trial, and Colony 2 and
469 Colony 5 for the second trial, along with the DWV source inoculum, buffer and unmanipulated
470 controls (12 pupae per replicate, 5 replicates per colony, see Figure 1 B for schematic). We
471 placed pupae into 0.6mL 96 well PCR plates so that we could monitor their development by

472 taking photographs of their eyes two times per day. We used a 1.5% w/v agar gel as a substrate
473 to maintain moisture and standardise the height of pupae, and added 0.01g 100mL⁻¹ copper
474 sulphate to the hot agar prior to pouring in order to inhibit fungal growth. We photographed
475 pupae using a Nikon D5100 camera with a Tamron 60mm F/2 macro lens and terminated the
476 experiment at day 6 prior to eclosion.

477

478 *3.1 Statistical analysis – survival screen*

479 We compared the pupae's survival with Cox's proportional hazards survival analyses using R-
480 3.3.3⁴⁵ with the package survival⁴⁶. We checked the log-linearity of covariates by plotting the
481 Cox models' martingale residuals against fitted values⁴⁷. We checked the proportional hazards
482 assumption of the Cox regression models following Grambsch & Therneau⁴⁸ (all $p > 0.05$). In
483 each Cox model we investigated for each colony the effect of 'treatment' (i.e. control, buffer,
484 pure DWV, serially transmitted BQCV and SBV, serially transmitted BQCV, SBV and DWV)
485 on pupae survival. We also included 'replicate' as a covariate, as well as the interaction between
486 treatment and replicate in all models. Since this interaction proved to be non significant in both
487 models (respectively colony 1: $p = 0.214$ and colony 2: $p = 0.244$), we removed the interaction
488 and recalculated the model. Post-hoc p -values were corrected for multiple comparisons
489 following the Benjamini and Hochberg procedure⁴⁹.

490

491 *Quarantine permits*

492 Frozen worker honey bee samples containing Deformed wing virus were imported from the
493 New Zealand Institute for Plant and Food Research, Hamilton, under our Department of
494 Agriculture and Water Resources import permit 0000917783. To work with DWV in Australia,
495 we require a quarantine permit that restricts us from injecting imported viruses into adult bees.
496 However, we are permitted to inject into pupae provided we terminate experiments prior to
497 eclosion. Therefore, we injected inoculate into white-eyed pupae, and we terminated each cycle
498 after 4 days to enable sufficient time for viral replication, while avoiding eclosion (Figure 1 A).

499

500 *Data Availability*

501 The raw sequencing reads from this project have been deposited to Genbank under the
502 Bioproject ID PRJNA397460 at the Sequence Read Archive (SRA Study ID: SRP114989). The
503 reference SBV, BQCV and source DWV sequences used in this study have been deposited to
504 Genbank under accession numbers MF623170, MF623171 and MF623172.

505

506

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513

514 **Author contributions**

515 EJР and MB designed the experiments. EJР, NM, TLG and BY performed the experiments.
516 EJР, NM, TLG, BY and MB analysed the data. EJР and MB wrote the paper.

517

518 **Competing financial interests**

519 The authors declare no competing financial interests

520

521 **References**

- 522 1 Carreck, N. L. & Ratnieks, F. L. W. Will neonicotinoid moratorium save the bees?
523 *Res. Fortnight* **415**, 20 and 22 (2013).
- 524 2 Cornman, R. S. *et al.* Pathogen webs in collapsing honey bee colonies. *PLoS One* **7**,
525 e43562, doi:doi:10.1371/
526 journal.pone.0043562 (2012).
- 527 3 Oldroyd, B. P. Coevolution while you wait: *Varroa jacobsoni*, a new parasite of
528 western honeybees. *Trends Ecol. Evol.* **14**, 312-315 (1999).
- 529 4 Ritter, W. *Varroa* disease of the honeybee *Apis mellifera*. *Bee World* **62**, 141-153
530 (1981).
- 531 5 Neumann, P. & Carreck, N. L. Honey bee colony losses. *J. Api. Res.* **49**, 1-6 (2010).
- 532 6 Martin, S. J. The Role of *Varroa* and Viral Pathogens in the Collapse of Honeybee
533 Colonies: A Modelling Approach. *J. Appl. Ecol.* **38**, 1082-1093 (2001).
- 534 7 Sumpter, D. J. T. & Martin, S. J. The dynamics of virus epidemics in *Varroa* infested
535 honey bee colonies. *J. Anim. Ecol.* **73**, 51-63 (2004).
- 536 8 Chen, Y. P. & Siede, R. Honey bee viruses. *Adv. Virus Res.* **70**, 33-80 (2007).

- 537 9 Martin, S. J. *et al.* Global honey bee viral landscape altered by a parasitic mite.
538 *Science* **336**, 1304-1306 (2012).
- 539 10 Mondet, F., Miranda de, J. R., Kretzschmar, A., Le Conte, Y. & Mercer, A. R. On the
540 front line: quantitative virus dynamics in honeybee (*Apis mellifera* L.) colonies
541 along a new expansion front of the parasite *Varroa destructor*. *PLoS Path.* **10**,
542 e1004323, doi:doi:10.1371/journal.ppat.1004323 (2014).
- 543 11 Ewald, P. W. Host-parasite relations, vectors, and the evolution of disease severity.
544 *Ann. Rev. Ecol. Syst.* **14**, 465-485 (1983).
- 545 12 Anderson, R. M. & May, R. M. Coevolution of hosts and parasites. *Parasitology* **85**,
546 411-426 (1982).
- 547 13 Frank, S. A. Models of parasite virulence. *Quart. Rev. Biol* **71**, 37-78 (1996).
- 548 14 Le Clec'h, W., Dittmer, J., Raimond, M., Bouchon, D. & Sicard, M. Phenotypic shift in
549 *Wolbachia* virulence towards its native host across serial horizontal passages.
550 *Proceedings of the Royal Society B: Biological Sciences* **284**,
551 doi:10.1098/rspb.2017.1076 (2017).
- 552 15 Ribiere, M., Ball, B. V. & Aubert, M. in *Virology and the honey bee* (eds M. Aubert,
553 B.V. Ball, & I. Fries) 15-84 (EEC Publications, European Commission, 2008).
- 554 16 Miranda de, J. R., Cordoni, G. & Budge, G. The Acute bee paralysis virus-Kashmit bee
555 virus-Israeli acute paralysis virus complex. *J. Invert. Path.* **103**, S30-S47 (2010).
- 556 17 Natsopoulou, M. E. *et al.* The virulent, emerging genotype B of Deformed wing virus
557 is closely linked to overwinter honeybee worker loss. *Scientific Reports* **7**, 5242,
558 doi:10.1038/s41598-017-05596-3 (2017).
- 559 18 Schroeder, D. C. & Martin, S. J. Deformed wing virus: The main suspect in
560 unexplained honeybee deaths worldwide. *Virulence* **3**, 589-591,
561 doi:10.4161/viru.22219 (2012).

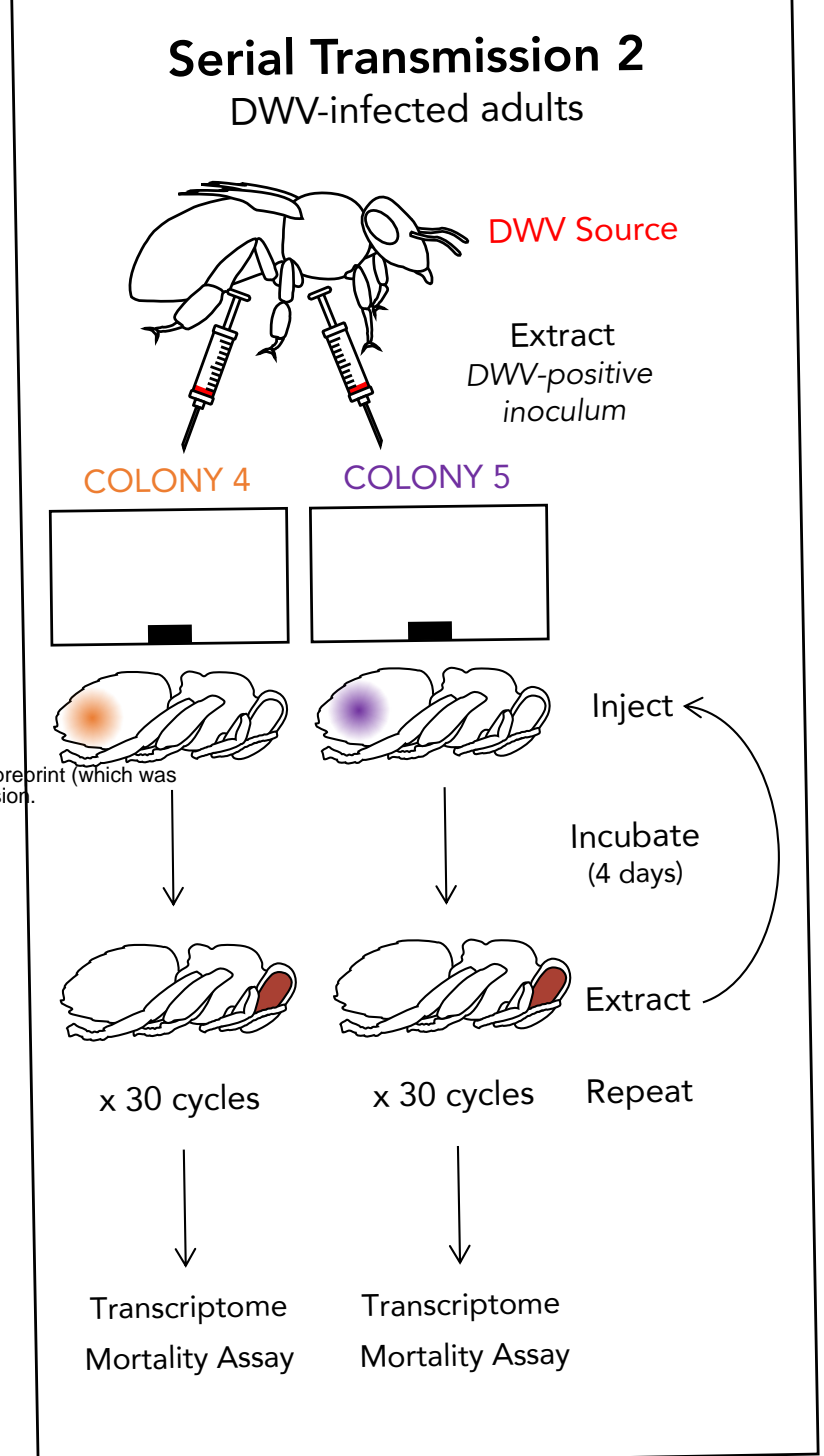
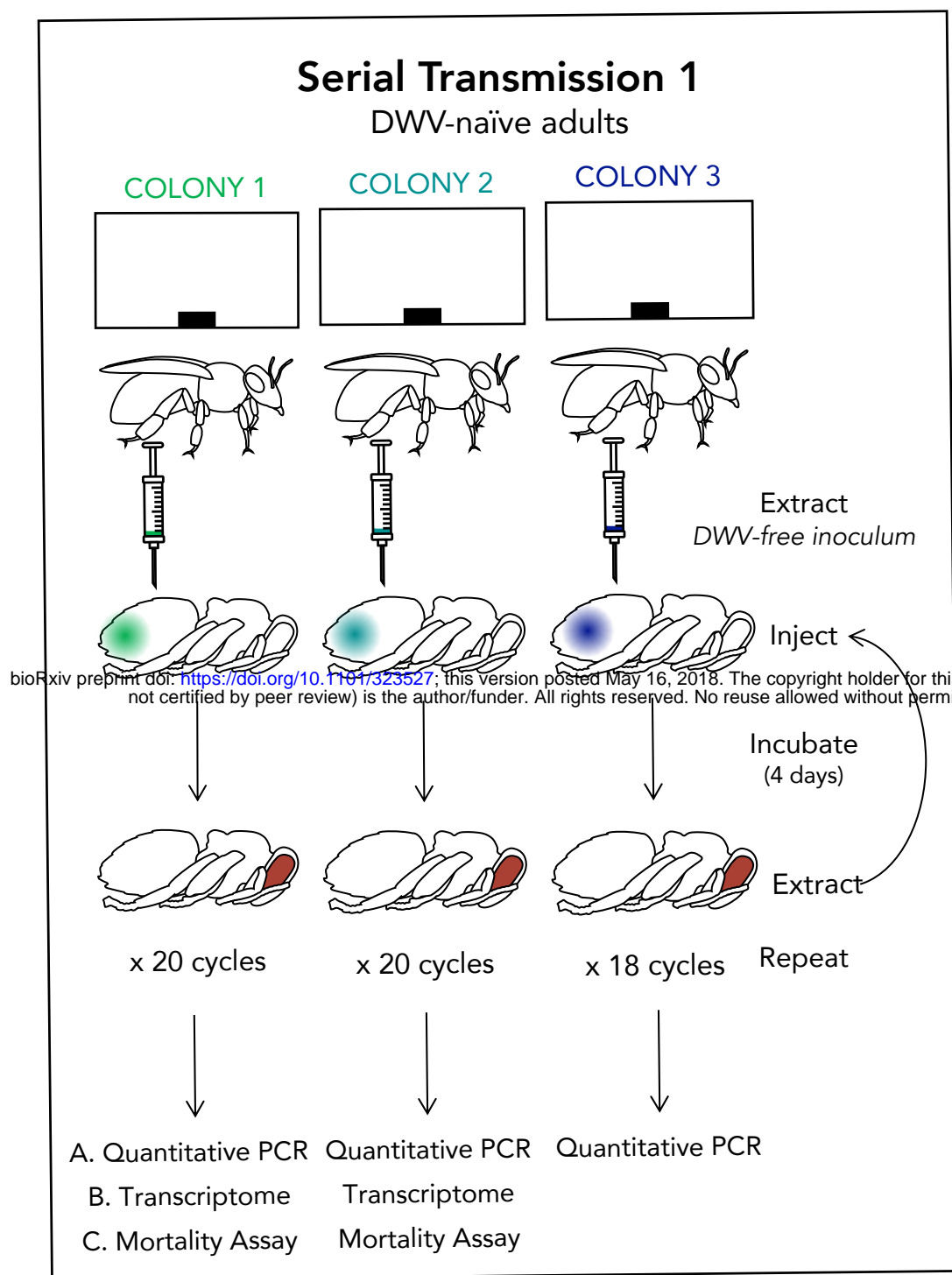
- 562 19 Anderson, D. L. & Gibbs, A. J. Inapparent virus infections and their interactions in
563 pupae of the honey bee (*Apis mellifera* Linnaeus) in Australia. *J. Gen. Virol.* **69**,
564 1617-1625 (1988).
- 565 20 McMahon, D. P. *et al.* Elevated virulence of an emerging viral genotype as a driver
566 of honeybee loss. *Proc. R. Soc. Lond. B* **283**, 20160811,
567 doi:<http://dx.doi.org/10.1098/rspb.2016.0811> (2016).
- 568 21 Roberts, J. M. K., Anderson, D. L. & Durr, P. A. Absence of deformed wing virus and
569 *Varroa destructor* in Australia provides unique perspectives on honeybee viral
570 landscapes and colony losses. *Scientific Reports* **7**, 6925, doi:10.1038/s41598-017-
571 07290-w (2017).
- 572 22 Mordecai, G. J. *et al.* Superinfection exclusion and the long-term survival of honey
573 bees in *Varroa*-infested colonies. *The ISME Journal* **10.1038/ismej.2015.186**, 1-
574 10, doi:10.1038/ismej.2015.186 (2015).
- 575 23 Mordecai, G. J., Wilfert, L., Martin, S. J., Jones, I. M. & Schroeder, D. C. Diversity of a
576 honey bee pathogen: first report of a third master variant of the Deformed Wing
577 Virus quasispecies. *The ISME Journal* **10.1038/ismej.2015.178**, 1-10,
578 doi:10.1038/ismej.2015.178 (2015).
- 579 24 Wilfert, L. *et al.* Deformed wing virus is a recent global epidemic in honeybees
580 driven by *Varroa* mites. *Science* **351**, 594-597 (2016).
- 581 25 Bailey, L. The multiplication and spread of sacbrood virus of bees. *Annals of Applied*
582 *Biology* **63**, 483-491, doi:10.1111/j.1744-7348.1969.tb02844.x (1969).
- 583 26 Bailey, L., Ball, B. V. & Perry, J. N. The prevalence of viruses of honey bees in Britain.
584 *Annals of Applied Biology* **97**, 109-118, doi:10.1111/j.1744-7348.1981.tb02999.x
585 (1981).

- 586 27 Bailey, L. & Woods, R. D. Two More Small RNA Viruses from Honey Bees and
587 Further Observations on Sacbrood and Acute Bee-Paralysis Viruses. *J. Gen. Virol.*
588 **37**, 175-182, doi:doi:10.1099/0022-1317-37-1-175 (1977).
- 589 28 Tentcheva, D. *et al.* Prevalence and seasonal variations of six bee viruses in *Apis*
590 *mellifera* L. and *Varroa destructor* mite populations in France. *Appl. Env. Microbiol.*
591 **70**, 7185-7191 (2004).
- 592 29 Holmes, E. C. The evolutionary genetics of emerging viruses. *Ann. Rev. Ecol. Evol.*
593 *Syst.* **40**, 353-372 (2009).
- 594 30 Ebert, D. Experimental evolution of parasites. *Science* **282**, 1432-1435 (1998).
- 595 31 Baalen van, M. & Sabelis, M. W. The dynamics of multiple infection and the
596 evolution of virulence. *Am. Nat.* **146**, 881-910 (1995).
- 597 32 Brettell, L. E. & Martin, S. J. Oldest *Varroa* tolerant honey bee population provides
598 insight into the origins of the global decline of honey bees. *Scientific Reports* **7**,
599 45953, doi:10.1038/srep45953
600 <https://www.nature.com/articles/srep45953#supplementary-information>
601 (2017).
- 602 33 Roberts, J. M. K., Anderson, D. L. & Durr, P. A. Metagenomic analysis of *Varroa*-free
603 Australian honey bees (*Apis mellifera*) shows a diverse *Picornavirales* virome. *J.*
604 *Gen. Virol.* doi:DOI 10.1099/jgv.0.001073 (2018).
- 605 34 Roberts, J. & Anderson, D. Establishing the disease status of the Asian honeybee in
606 the Cairns region. (2013).
- 607 35 Pfaffl, M. W., Tichopad, A., Prgomet, C. & Neuvians, T. P. Detrmination of stable
608 housekeeping genes, differentially regulated target genes and sample integrity:
609 BestKeeper - Excel-based tool using pair-wise correlations. *Biotech. Lett.* **26**, 509-
610 515 (2004).

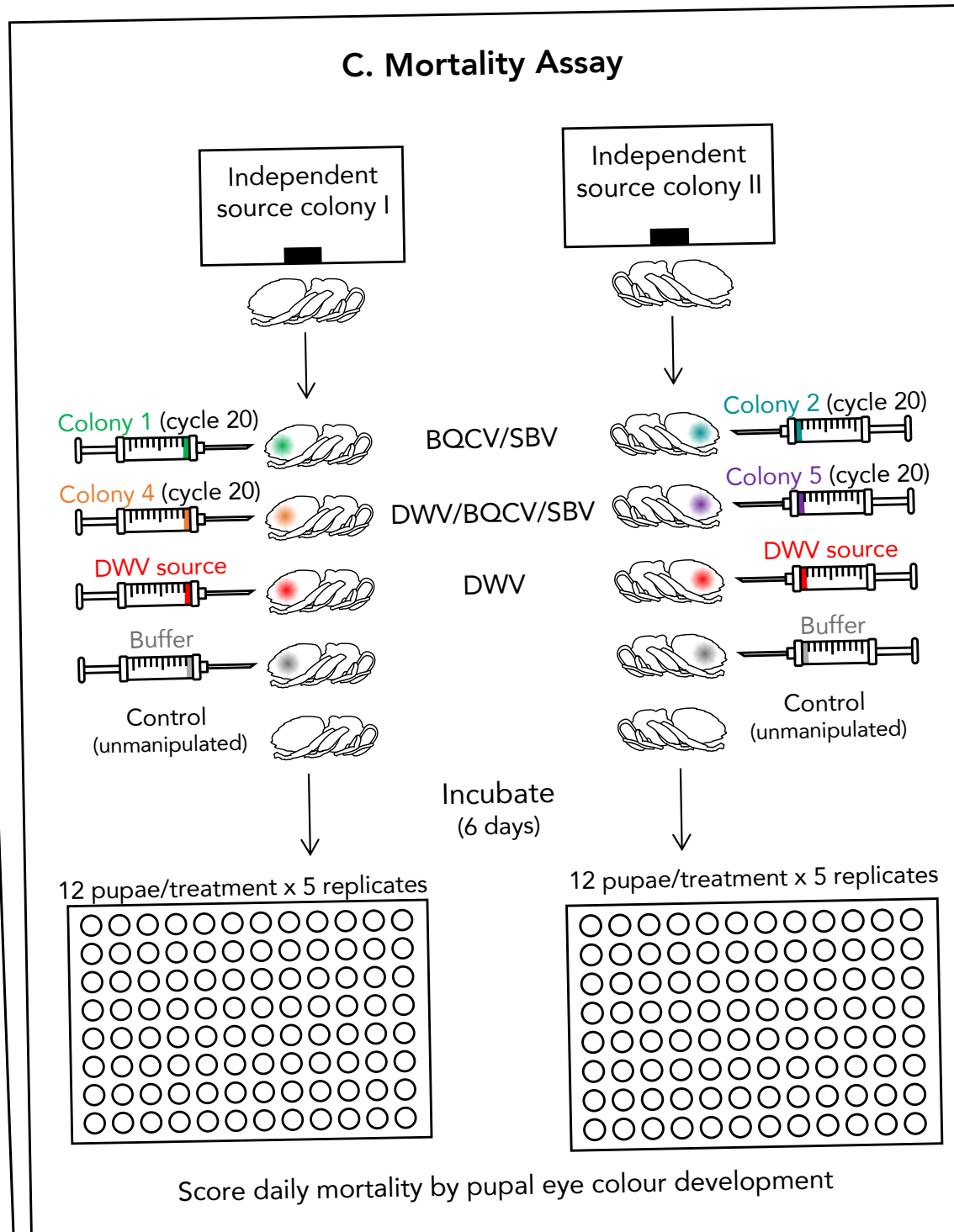
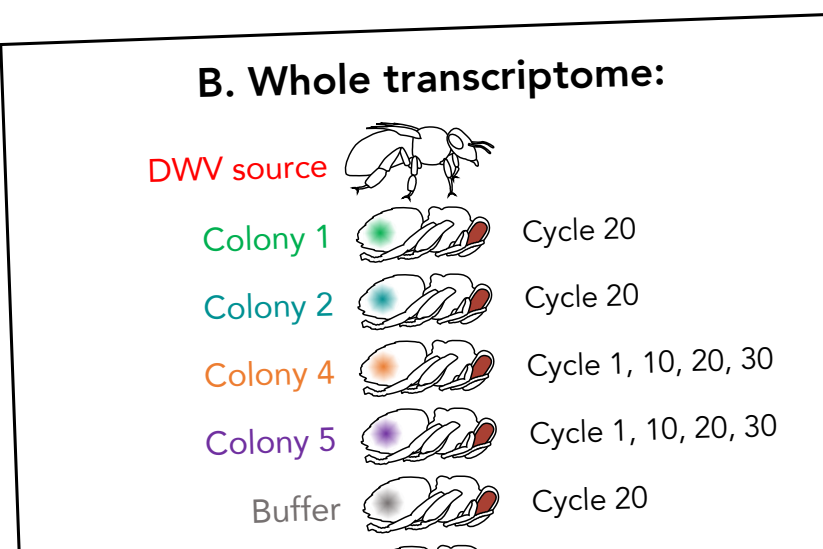
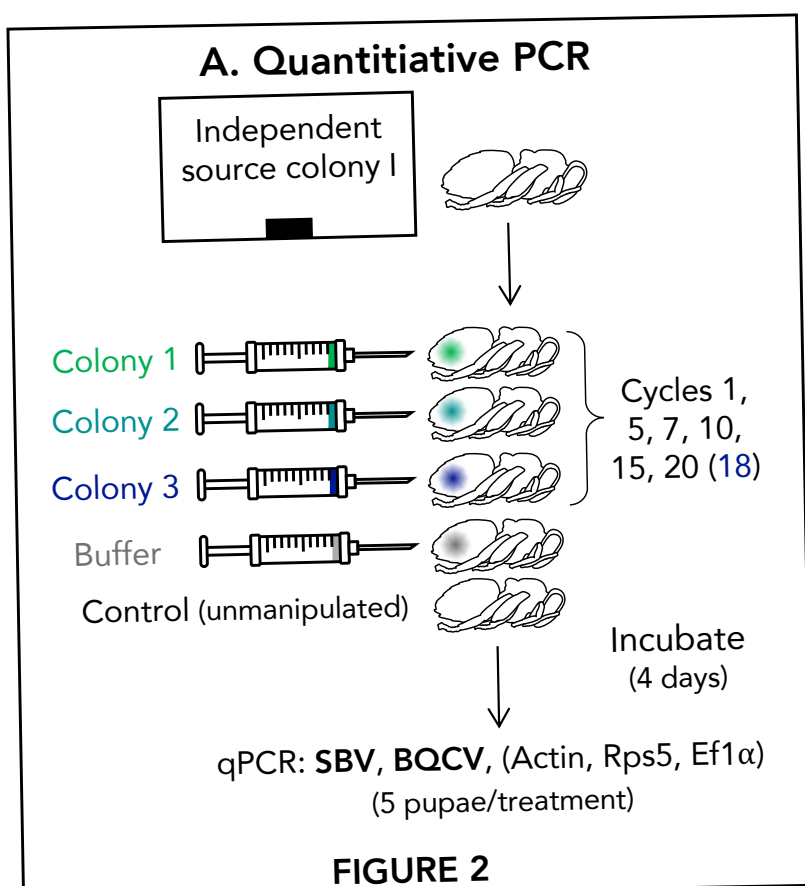
- 611 36 Vandesompele, J. *et al.* Accurate normalization of real-time quantitative RT-PCR
612 data by geometric averaging of multiple internal control genes. *Gen. Biol.* **3**,
613 research0034.0031–0034.0011,
614 doi:<http://genomebiology.com/2002/3/7/research/0034.1> (2002).
- 615 37 Grabherr, M. G. *et al.* Full-length transcriptome assembly from RNA-Seq data
616 without a reference genome. *Nature Biotech.* **29**, 644–652 (2011).
- 617 38 Remnant, E. J. *et al.* A diverse range of novel RNA viruses in geographically distinct
618 honey bee populations. *J. Virol.* **91**, e00158-00117, doi:10.1128/JVI.00158-17
619 (2017).
- 620 39 Kircher, M., Sawyer, S. & Meyer, M. Double indexing overcomes inaccuracies in
621 multiplex sequencing on the Illumina platform. *Nucleic Acids Research* **40**, e3
622 (2012).
- 623 40 Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nature*
624 *Methods* **9**, 357–359 (2012).
- 625 41 Ghosh, R. C., Ball, B. V., Willcocks, M. M. & Carter, M. J. The nucleotide sequence of
626 sacbrood virus of the honey bee: an insect picorna-like virus. *J. Gen. Virol.* **80**, 1541-
627 1549 (1999).
- 628 42 Leat, N., Ball, B., Govan, V. & Davison, S. Analysis of the complete genome sequence
629 of black queen-cell virus, a picorna-like virus of honey bees. *J. Gen. Virol.* **81**, 2111-
630 2119 (2000).
- 631 43 Kearse, M. *et al.* Geneious Basic: An integrated and extendable desktop software
632 platform for the organization and analysis of sequence data. *Bioinformatics* **28**,
633 1647–1649, doi:10.1093/bioinformatics/bts199 (2012).
- 634 44 Dustmann, J. H. Pigment Studies on Several Eye-colour Mutants of the Honey Bee,
635 *Apis mellifera*. *Nature* **219**, 950–952 (1968).

- 636 45 Team, R. C. R: A language and environment for statistical computing. *R Foundation*
637 *for Statistical Computing, Vienna, Austria* (2017).
- 638 46 Therneau, T. A package for survival analysis in S. . *version 2.38*, [https://CRAN.R-](https://CRAN.R-project.org/package=survival)
639 [project.org/package=survival](https://CRAN.R-project.org/package=survival) (2015).
- 640 47 Fox, J. Cox proportional-hazards regression for survival data. *An R and S-PLUS*
641 *companion to applied regression* (2002).
- 642 48 Grambsch, P. M. & Therneau, T. M. Proportional hazards tests and diagnostics based
643 on weighted residuals. *Biometrika* **81**, 515-526 (1994).
- 644 49 Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate - a practical and
645 powerful approach to multiple testing. *Journal of the Royal Society Statistical*
646 *Society Series B - Statistical Methodology* **57**, 289-300 (1995).
- 647
- 648
- 649

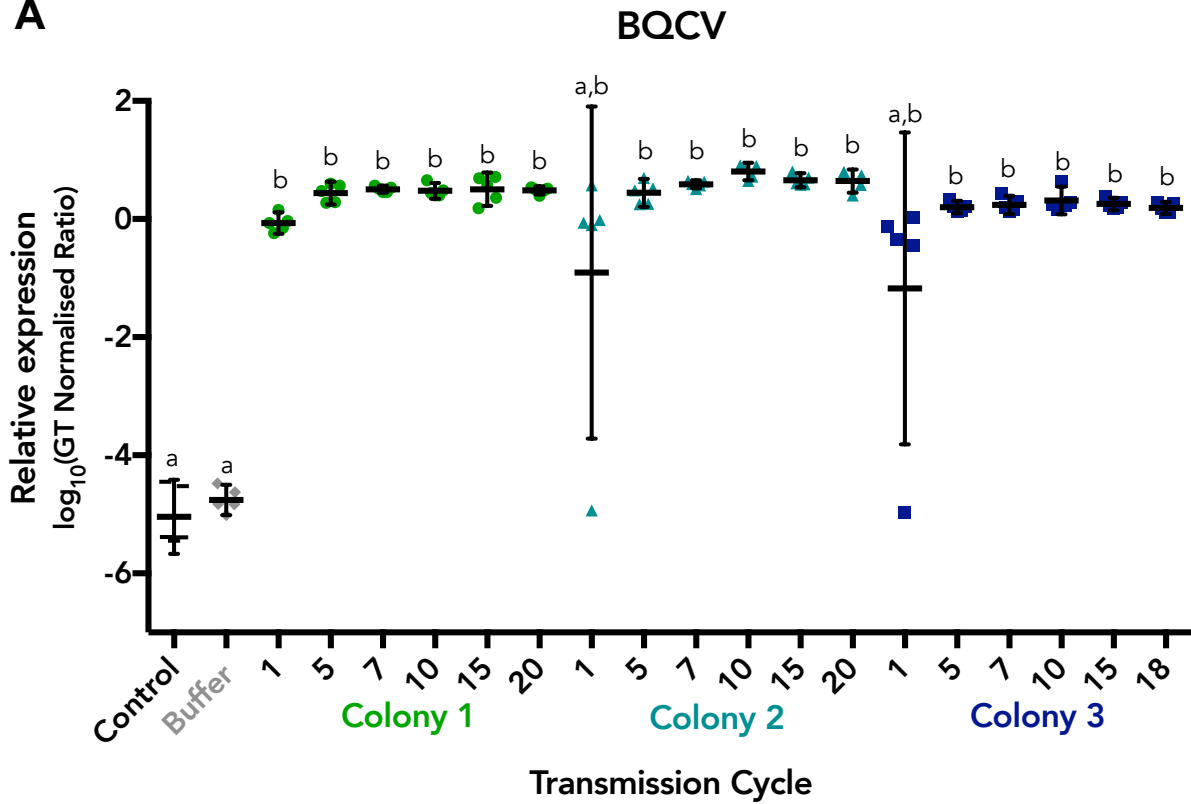
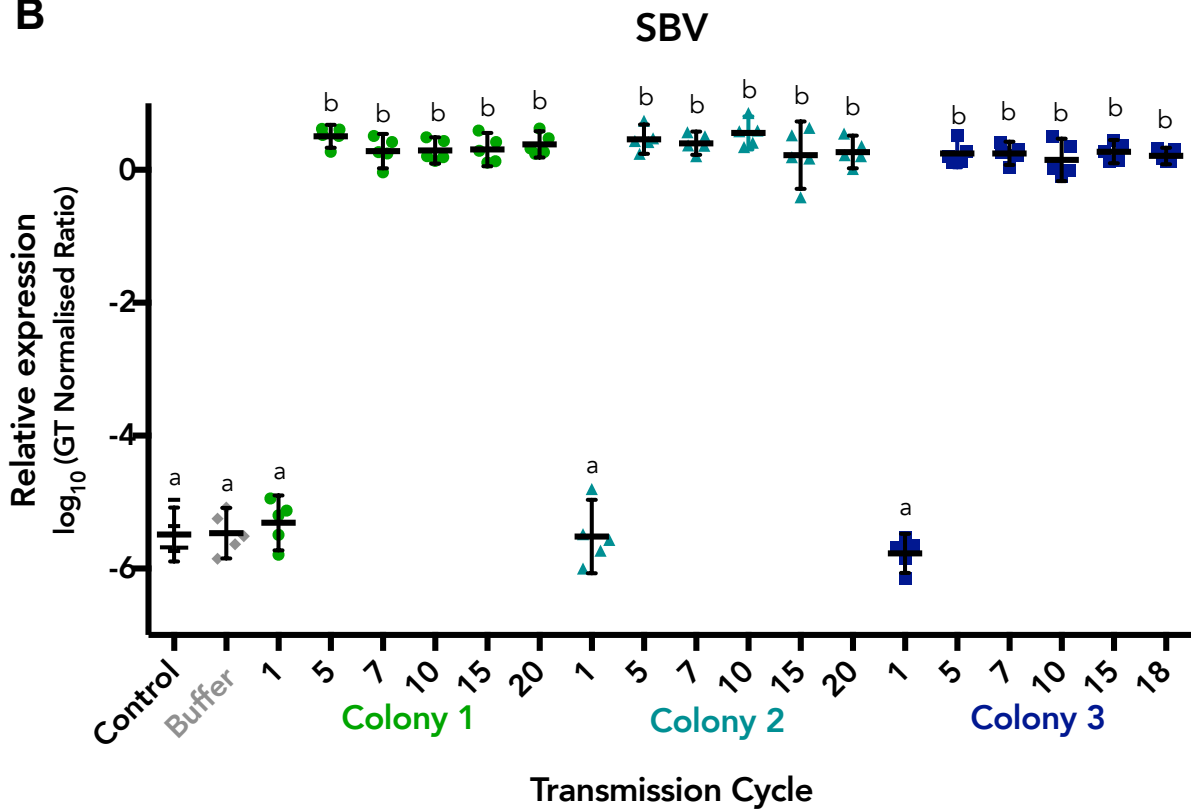
650 **Figure 1:** Experimental design of serial transmission experiments. **(A)** Serial transmission 1,
651 with starting inoculum derived from DWV-naïve adults, and injected into pupae from Colonies
652 1-3 for 20 serial transmission cycles. The number of cycles differed for colony 3 as this colony
653 lost its queen after 18 cycles. Serial transmission 2, with starting inoculum derived from DWV-
654 positive adults from New Zealand, injected into pupae from colonies 4-6. Colony 6 lost its
655 queen early on in the experiment; hence this colony was not included in any further analyses.
656 **(B)** Resulting virus levels and virulence were determined by quantitative PCR (see results in
657 Figure 2), whole transcriptome sequencing (see Figure 3) and mortality assays (see figure 4,
658 and text for further details)
659



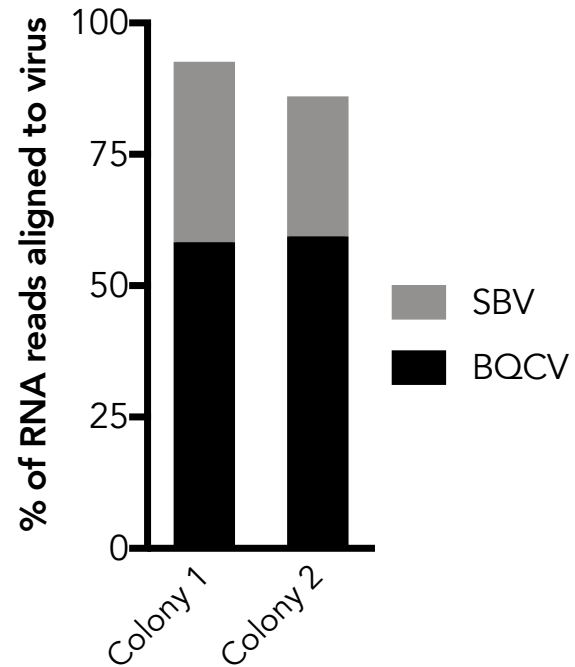
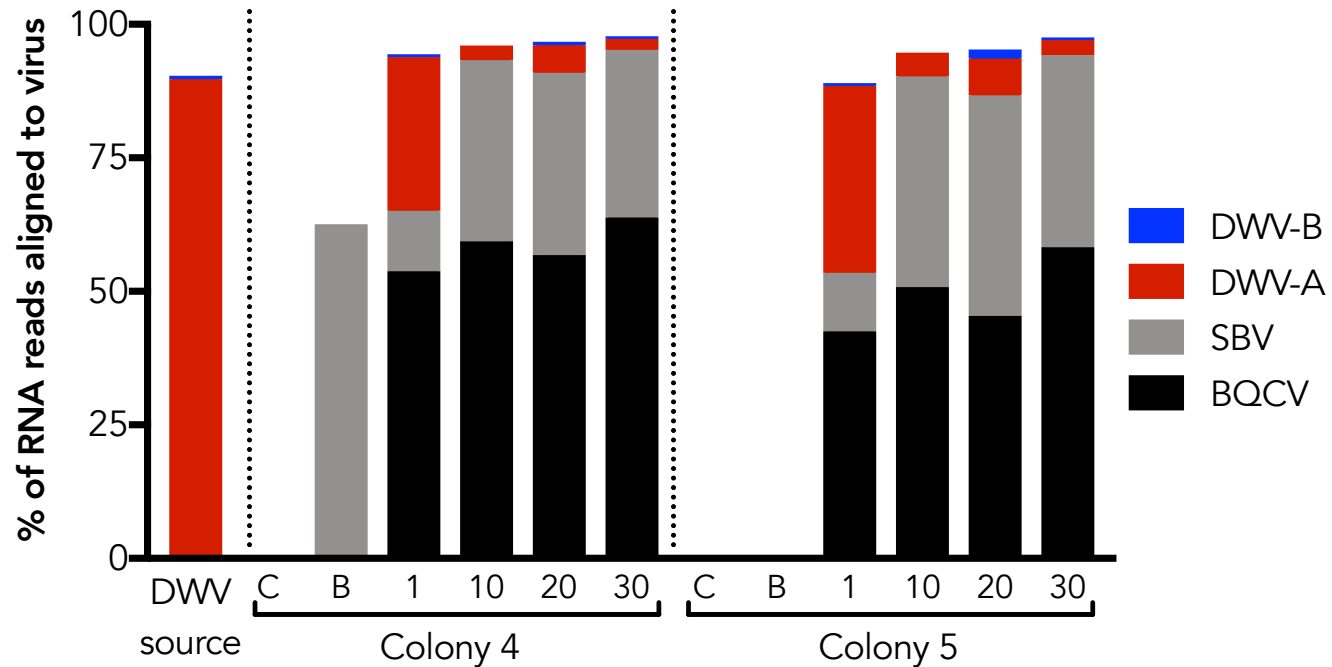
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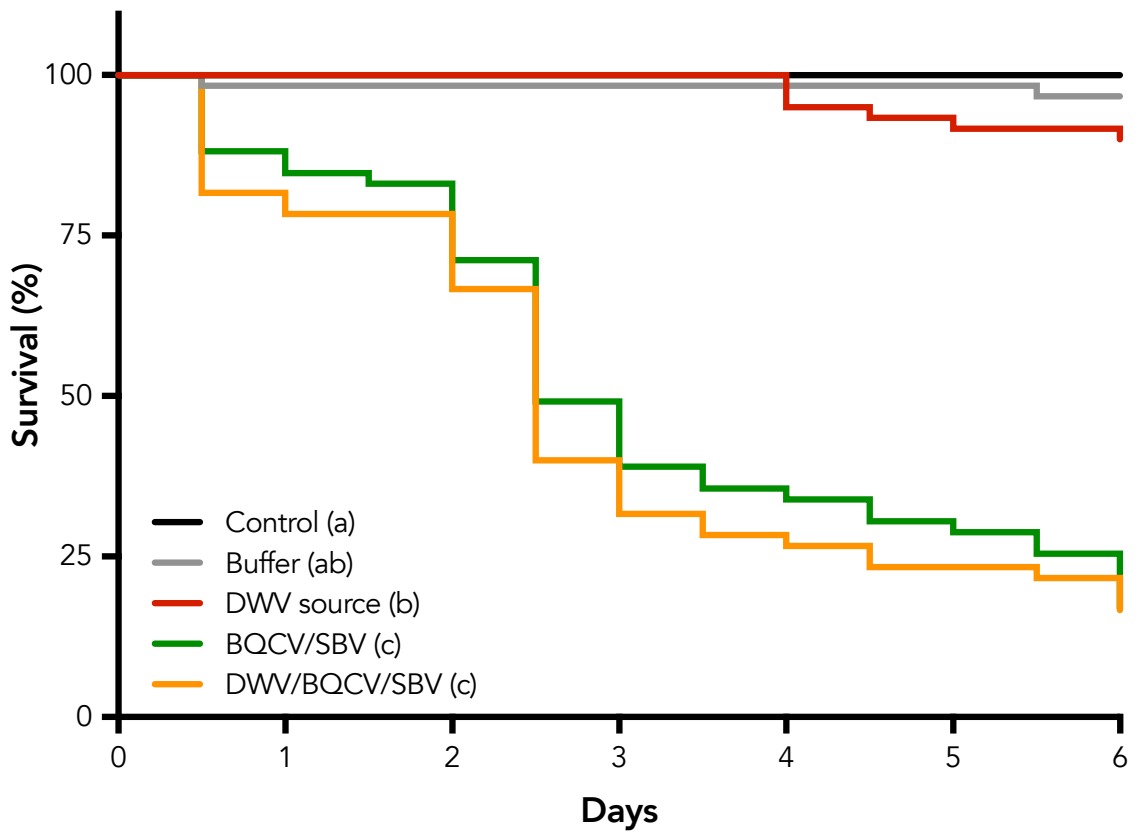
660 **Figure 2:** Dot plots showing the relative expression level ($\log_{10}(\text{GT Normalised Ratio})$, mean
661 $\pm 95\%$ CI) of **(A)** BQCV and **(B)** SBV compared to two internal honeybee control genes (*Actin*
662 and *Rps5*) in pupae sourced from an independent colony and injected with serially transmitted
663 inoculum from colonies 1-3 (transmission cycles 1, 5, 7, 10, 15 and 18 or 20), and control and
664 buffer injected pupae. Letters indicate which groups differed statistically. See Table S2 for
665 details of the statistical analyses.
666

A**B**

667 **Figure 3:** Change in viral titres during (A) serial transmission experiment 1 (DWV-naïve); and
668 (B) serial transmission experiment 2 (DWV-positive). A) Levels of SBV (grey) and BQCV
669 (black) in pupae from colony 1 and 2, 4-days post injection with inoculum after 20 serial
670 transmission cycles. Virus levels reached 92 and 86% as a percentage of total RNA,
671 respectively. B) Levels of SBV, BQCV and DWV-strain A (red) and strain-B (blue) in our
672 original inoculum obtained from DWV-positive adults (DWV source), and pupae from colony
673 4 and 5, 4-days post injection with inoculum after 1, 10 and 20 serial transmission cycles. Also
674 shown are control and buffer pupae from transmission cycle 20. Although our original inoculum
675 ('Source (DWV)') contained exclusively DWV, DWV titres dropped dramatically after
676 injection into pupae, while the titres of SBV and BQCV increased. While our original inoculum
677 mainly contained DWV strain A; the contribution of strain B had increased after 20
678 transmission cycles, particularly in colony 5, but decreased again after 30 transmission cycles.
679 Data used to produce the figure are presented in Table S3.
680

A**B**

681 **Figure 4:** Survival of pupae after injections with inoculum obtained from our original source
682 adults (DWV source) containing mainly DWV strain A, and inoculum extracted after 20
683 transmission cycles in the absence and presence of DWV, from serial transmission experiment
684 1 (BQCV/SBV) and serial transmission experiment 3 (DWV/BQCV/SBV). Treatments with
685 the same letter do not differ significantly (see Table 1 for complete statistical analyses). Virus
686 levels present in the different inoculums are given in Figure 3. ‘Control’: unmanipulated pupae;
687 ‘Buffer’: pupae injected with buffer only. **(A)** BQCV/SBV inoculum from cycle 20 of colony
688 1; DWV/BQCV/SBV inoculum from cycle 20 of colony 4, injected into independent source
689 colony I; **(B)** BQCV/SBV inoculum from cycle 20 of colony 2; DWV/BQCV/SBV inoculum
690 from cycle 20 of colony 5, injected into independent source colony II. See Figure S2 for
691 photographs depicting the eye colour change observed in a control and bee extract-injected
692 pupa, used to determine time of mortality in the survival analysis.

A**B**