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A rapid survey technique for *Tropilaelaps* mite (Mesostigmata: Laelapidae) detection

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Abstract

Parasitic mites harm pollinator health; the Varroa mite (*Varroa destructor* Anderson and Trueman) is the most serious single threat to honey bees. Another group of mites with similar life histories to Varroa mites, *Tropilaelaps* (Delfinado and Baker) species, have become a damaging pest of European honey bees (*Apis mellifera* L.) in Asia. These mites represent a significant threat if introduced to other regions of the world. The seriousness of this threat warrants implementation of Tropilaelaps mite surveillance in regions not thought to be infested. Current Tropilaelaps mite detection methods are unsuitable for efficient large scale screening. We thus developed and tested a new bump technique that consists of firmly rapping a brood frame from a honey bee hive over a collecting pan. Our method was easier to implement than current detection tests, reduced time spent in each apiary and avoided brood destruction. This feasibility increase overcomes the test's decrease in the probability of detecting infested colonies (sensitivity). Considering the sensitivity of the bump test, we suggest that screening programs sample seven colonies per apiary and 312 randomly selected apiaries in a region to be 95% sure of detecting an incipient Tropilaelaps mite invasion. Further analyses counter the currently-held view that Tropilaelaps mites prefer drone bee brood cells. We propose this test as a standard tool for monitoring of Tropilaelaps mite presence in regions thought to be free from infestation.

Key words: Tropilaelaps mite, surveillance, honey bee, screening protocols

45 The European honey bee (*Apis mellifera* L.), the most commonly used managed pollinator in the
46 U.S., pollinates over 100 North American commercial crops and directly contributes between 5
47 and 10 billion dollars annually to the U.S. economy (2005 adjusted $; NRC 2006). A decline in
48 honey bee health has been documented for 50 years (vanEngelsdorp and Meixner 2010), and
49 overwintering honey bee losses have been reported at approximately 30% annually in the U.S.
50 over the last 5 winters (vanEngelsdorp et al. 2008, vanEngelsdorp et al. 2010, vanEngelsdorp et
51 al. 2011, VanEngelsdorp et al. 2012). Managed colonies are at risk from several pests and
52 diseases, including parasitic mites. Currently, the Varroa mite (*Varroa destructor* Anderson and
53 Trueman) is thought of as the biggest threat to managed honey bees in the U.S. (Rosenkranz et
54 al. 2010, VanEngelsdorp et al. 2012). Other parasitic mites such as those in the genus
55 *Tropilaelaps* (Delfinado and Baker) cause significant losses in countries such as Thailand, the
56 Philippines and Pakistan (Camphor et al. 2005). In addition, Tropilaelaps mites are capable of
57 vectoring viruses, and may cause additional declines by interacting with Varroa mites (Dainat et
58 al. 2009, Sanpa and Chantawannakul 2009). Tropilaelaps mite invasion in the U.S. or Europe
59 would likely increased economic losses and the decline in honey bee health (Department for
60 Environment 2005). Thus, it is crucial to develop an effective Tropilaelaps mite surveying
61 method to allow early detection after potential introductions to regions outside of the mite's
62 natural range in Asia.

63 Tropilaelaps mites are honey bee ectoparasites that predominantly feed on developing
64 bees (bee brood, including larval and pupal stages). Parasitism by these mites can cause brood
65 mortality and colony decline (Ritter 2008). The sister species *Tropilaelaps clareae* Delfinado
66 and Baker and *Tropilaelaps mercedesae* Anderson and Morgan (henceforth collectively referred
67 to as Tropilaelaps mites) expanded their preferred hosts to include the European honey bee in

addition to the giant honey bee (*A. dorsata* F.) after the former was introduced to Asia. These mites are a major threat to managed European honey bees (Anderson and Morgan 2007). Tropilaelaps mites have a higher reproductive rate and shorter life cycle than Varroa mites, thus they may outcompete Varroa mites when both mites are present (Burgett et al. 1983, Ritter and Schneider-Ritter 1988). This rapid reproduction and recent geographic spread make Tropilaelaps mites an emerging threat to managed honey bees worldwide (Sammataro et al. 2000, Ritter 2008).

The U.S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) does not allow imports of bees from another nation that has a bee disease, parasite or pest not found in the U.S, including those where the Tropilaelaps mite is known to occur. Considering the serious threat these mites pose to the apicultural industry it is important not only to enforce laws which aim to prevent the mite's spread into the country, but also to have a surveillance system in place so that any introduction is quickly identified and can be eradicated. Several sampling methods have been previously described, including the use of sticky boards, an ether or sugar roll and visually inspecting brood cells (Ritter and Schneider-Ritter 1988, Sammataro et al. 2000, Koeniger et al. 2002, Ritter 2008). However, these sample methods may not be appropriate for large-scale screening if they are not sufficiently dependable in detecting infested colonies, or are so time consuming that they are impractical to implement on a large scale (Ritter and Akratanakul 2006).

The primary aim of this study was to develop a rapid Tropilaelaps mite detection technique aimed at early detection of mite invasions. Our goal was to develop a test that balanced the need for high sensitivity with limited surveillance resources. We tested previously described methods, and two new methods that employ a bumping technique, for economy of

implementation (time and effort) and *sensitivity* (correct detection of infected colonies). Surveys aim to detect mites with 95% confidence in apiaries that are suffering negative effects (Delaplane and Hood 1999, OIE 2012). However, screening methods also need to be quick with minimal visits to each tested apiary. Here we 1) describe the new bumping technique for Tropilaelaps mite detection and 2) compare its reliability to previously described detection methods. We then establish guidelines for effectively surveying apiaries with the bumping technique.

Materials and Methods

In September 2009, we evaluated Tropilaelaps mite sampling and detection techniques in ten apiaries in Chiang Mai, Thailand. Beekeepers in this area actively treat colonies once every two weeks to control Tropilaelaps and Varroa mite levels. Both mites are endemic to this area (Sammataro et al. 2000, Anderson and Morgan 2007), and can cause colony decline if left untreated.

In all colonies in each apiary, we quantified adult bee and brood populations to the nearest 0.5 standard brood nest frames, and sampled individual colonies for Tropilaelaps and Varroa mite presence in seven ways. Methods 1, 2, 4 and 5 are currently used to test for Tropilaelaps mite presence in Asia (Ritter 2008); method 3 has since been adopted by the USDA-APHIS national honey bee disease survey (Rennich et al. 2011).

1) Drop method: We placed screen-covered sticky boards under the comb of colonies for 24 hours, and then removed them and counted mites.

2) Wash method: Approximately 300 adult bees per colony were collected and stored in ethanol for later mite quantification using a modified soapy water shake method (Ritter 2008), where the

soapy water dislodges the mites from the bees. This method is currently used to measure varroa mite infestations. (Lee et al. 2010).

3) Bump method (new): All adult bees were removed from one frame containing capped brood by shaking the frame over the colony. Once adult bees were cleared away, we firmly bumped frames over a white metal pan by hitting one end of the frame on the side of the pan, turning the frame, re-bumping the frame and repeating the process once more for a total of four bumps. This process dislodged mites on the surface of the frame, which we then counted.

4) Worker Brood method: After removing adult bees as above, we visually surveyed mite presence by examining up to 100 worker brood cells (mean 95.6 cells/colony). All examined brood were in the post-larval stage. This method required uncapping cells by removing the wax covering, then removing the larvae and pupae for examination. This resulted in loss of the removed brood.

5) Drone Brood method: We used the worker brood detection test on drone brood, examining up to 20 drone brood cells (mean 16.9 cells/colony).

6) Post-Bump method (new): After examining brood, we bumped frames again and counted dislodged mites. The Post-Bump method was intended to determine whether uncapping cells and removing brood exposes more mites than simply bumping otherwise undisturbed frames (as in the Bump method).

7) Bald brood method: We noted whether each frame of capped brood examined with methods 4 and 5 contained cells that were fully capped or also contained bald brood (see Fig. 1). Local beekeepers suggested that *bald brood*, a condition where the pupal caps have been removed and the developing pupae are exposed (Villegas and Villa 2006), indicates heavy Tropilaelaps mite infection.

136 Initial data collection involved three apiaries with 24 to 40 colonies each. We then
137 expanded the study to an additional seven apiaries, sampling 19-22 of their 21-107 colonies.
138 After the initial three apiaries were surveyed, we discontinued the Drop (1) and Wash (2)
139 methods. They proved too difficult (as the mite is small – Fig. 2 – and was easily confused with
140 hive debris, Fig. 3), time-consuming or not predictive of mite presence (adult bee wash; see also
141 Waghchoure-Camphor and Martin 2009). For each detection method we calculated Tropilaelaps
142 and Varroa mites' *prevalence* (proportion of individuals or colonies in a population that are
143 infected) within an apiary and mean abundance per colony. Unless otherwise indicated the
144 population for prevalence values is the apiary, and prevalence indicates the proportion of
145 examined colonies in an apiary with detectable mite levels. We use a non-technical definition of
146 *abundance* to refer to all measures of the number of mites per individual bee or per colony,
147 because units vary across detection tests (Table 1).
148 We calculated the sensitivity for each method's ability to screen for Tropilaelaps and
149 Varroa mites. First we classified colonies as having known mite infestations with two different
150 standards. A) Infestation verified: At least one sampling method detected mites. This approach
151 assumes that the combination of sample methods would detect mites in all infested colonies. B)
152 Universal infestation assumed: We assumed that every colony in an apiary with some mites is
153 infested. Universal infestation is likely, since managed colonies in Tropilaelaps mites' native
154 range almost always become infested without mite control (Ritter and Akratanakul 2006) and
155 frequent interactions between honey bee colonies provide opportunities for inter-nest transfer of
156 adult mites (Evans and Schwarz 2011). For each method, we then calculated its sensitivity as the
157 percentage of colonies with known mite infestations in which it detected mites, separately
158 calculating sensitivity with standards A and B and for each mite type.

159 Next we investigated whether the bald brood condition is a reliable indicator of
160 Tropilaelaps mite presence. We quantified bald brood prevalence within each colony and
161 calculated this detection method's sensitivity under an assumed universal infestation (B). For
162 each type of mite, we then tested the relationship between colonies' bald brood and mite statuses
163 with a McNemar's test (Zar 1999), and bald brood status with Tropilaelaps mite abundance in
164 worker brood with a Wilcoxon signed-rank test (Zar 1999). Statistical analyses were conducted
165 in JMP (SAS Institute Inc. 2012).
166 To verify that selecting a random brood frame when testing for Tropilaelaps mite
167 infestation yields unbiased results, we measured mite infestations on two different brood frames
168 in 140 colonies, inspecting 50 worker and up to 20 drone brood (larvae and pupae) cells per
169 colony. We compared the proportion of cells examined on each frame that were mite infested
170 with a Wilcoxon signed-rank test. We also compared infestation abundance in pre-pupal vs.
171 pupal cells and drone vs. worker brood cells via Wilcoxon signed-rank tests. Previous results
172 suggest Tropilaelaps mites prefer drone brood (Burgett et al. 1983, but see Waghchoure-
173 Camphor and Martin 2009).
174 We tested patterns of Tropilaelaps mite-Varroa mite co-infestation to determine if they
175 vary from those expected by chance by comparing infestation abundances measured with the
176 Worker Brood (4) and Drone Brood (5) methods. Deviations from the expected co-infestation
177 rate suggest facilitation or competition between the two types of mites.
178 Finally, we used Tropilaelaps mite within-colony infestation levels and test sensitivities
179 to develop practical guidelines for large-scale screening of apiaries with our bumping technique.
180 First we determined whether colonies with higher infestation abundance (proportion of examined
181 worker brood cells with Tropilaelaps mites) were more likely to test positive for these mites. We

182 classified each colony's infestation abundance as having Tropilaelaps mites in 0%, 0.1–4.5% or
183 >4.5% of the worker brood cells inspected, then separately calculated detection test sensitivities
184 (universal infestation assumption) within each category. Our relatively low infestation levels
185 reflect the fact that all beekeepers whose apiaries we used regularly treat their colonies with
186 acaricides (mainly sulfur, naphthalene and amitraz) to prevent colony mortality. Second, we
187 determined the number of colonies that need to be examined in an apiary to detect Tropilaelaps
188 mites. Because false positives are not possible with our detection methods, we could not use
189 standard epidemiological methods (e.g., positive predictive value) that rely on false positive
190 rates. We thus calculated the probability of detecting Tropilaelaps mites in at least one colony of
191 an infested apiary with the Bump method by assuming that Tropilaelaps mites spread randomly
192 within an apiary. The number of infested colonies within an apiary thus follows a binomial
193 distribution (Culliney 2003) with the following parameters: n = number of colonies tested, X =
194 number of the n colonies that test positive, and p = probability that a sampled colony tests
195 positive. Testing positive requires that both the colony is infested and the test detects that
196 infestation, so p = prevalence (% of colonies in the apiary assumed infested) * test sensitivity.
197 Thus P_{pos}, the probability that screening detects at least one Tropilaelaps-positive colony when
198 sampling n colonies per apiary, is:

199 $$P_{pos} = 1 - \mathrm{P}(\text{detect 0 Tropilaelaps-positive colonies})$$

200 $$= 1 - \mathrm{P}(X=0)$$

201 $$= 1 - \binom{n}{0} p^0 (1-p)^{n-0}$$

202 $$= 1 - (1-p)^n$$

203 $$= 1 - (1 - prevalence * sensitivity)^n$$

204 Thus, $n_{colonies} = \frac{\ln(1 - P_{pos})}{\ln(1 - prevalence * sensitivity)}$

205 We used the most conservative Bump test sensitivity figure — 36% assuming universal
206 infestation — to determine the smallest number of colonies that should be tested per apiary to
207 reach our goal of detecting mites with 95% confidence (P_{pos} = 0.95).

208 This same equation can be applied to a region or zone within a country to determine the
209 number of apiaries that must be randomly sampled to detect at least one Tropilaelaps mite-
210 positive apiary at the start of an invasion. Here, prevalence represents the proportion of apiaries
211 in a region assumed to be infested. This number will be very low at the start of an invasion.
212 Sensitivity now refers to the probability that bumping $n_{colonies}$ colonies per apiary detects an
213 infested apiary. This value is P_{pos} from above, or 0.95. We used this and several prevalence
214 values to calculate $n_{apiaries}$, the smallest number of apiaries that should be tested to detect a
215 Tropilaelaps mite invasion with 95% confidence.

216

217 **Results**

218 **Tests' Performance**

219 The 236 colonies examined averaged 5.6 ± 0.09 frames of adult bees (range 2 - 8) and 4.4
220 ± 0.09 frames of brood (1 - 7). Our methods detected Tropilaelaps mite infestations in 74.6% of
221 the colonies and 100% of the apiaries examined. Infestation prevalence varied dramatically,
222 averaging from only 4.8% of an apiary with the Wash test to approximately half or more of the
223 colonies in an apiary having Tropilaelaps mites with the Drop, Worker Brood or Post-Bump tests
224 (Table 1). Mite infestation abundance units vary by test, and thus are not directly comparable.
225 However, all tests detected, on average, at least one Tropilaelaps mite per colony and most
226 detected several (Table 1).

227 With the exception of the low-sensitivity Drone Brood method, our bumping technique
228 was the least time-intensive detection test (Table 2). The Drop test required two visits on
229 consecutive days to each apiary. The Worker Brood test took the longest to implement since it
230 required removing brood from cells and scanning for mites. The Worker Brood, Drone Brood
231 and Post-Bump tests also required exposing brood for an extended period of time, and destroying
232 brood.

233 Bumping frames to test for Tropilaelaps mite presence performed better than two
234 standard detection methods (Wash and Drone Brood) but worse than others (Drop, Worker
235 Brood). The sticky board was the most sensitive test, correctly identifying 81.3% of colonies
236 known to have a Tropilaelaps mite infestation (standard A). If a universal infestation of mites is
237 assumed (standard B), examination of worker brood was the most sensitive (56.7%; Table 1).
238 The least sensitive test was the adult bee wash, which detected only 7.8% of cases with verified
239 Tropilaelaps mite infestation and 5.2% of cases when universal infestation was assumed.
240 Bumping before removing brood cells (Bump test) was less sensitive than post-removal bumping
241 under both assumptions (50.0% vs. 61.5% with known Tropilaelaps mite infestations, 36.3% vs.
242 49.1% assuming universal infestation).

243 We found Varroa mites in 66.5% of the colonies, and 100% of the apiaries, with at least
244 one of the detection techniques. The current test for Varroa mite presence, washing adult bees,
245 had fairly low sensitivity (Table 1). Our Bump test was even less sensitive, detecting only 10.3%
246 of infested colonies with a verified infection and 6.5% assuming universal infestation. However,
247 Varroa mite abundances were fairly low in most of the examined colonies (Table 1). The more
248 time-consuming or intrusive tests exhibited higher sensitivities.

249 Bald brood occurred in 62.7% of the colonies examined, and was a fairly sensitive test
250 for Tropilaelaps and Varroa mites (50.9 and 39.8%, respectively). In total 7.3% of the 23,305
251 worker cells examined were classified as bald. Colonies with bald brood were 1.3 times more
252 likely to test positive for Tropilaelaps mites under at least one detection test than those with only
253 capped worker brood (81.1% vs. 63.6%; McNemar's test: χ^2=9.3, df=1, *P*=0.002). Tropilaelaps
254 mite abundances were significantly higher in bald (23.4% ± 2.5%) than capped cells (3.9% ±
255 0.4%; Wilcoxon signed-rank test: n = 141 colonies, W = 2,043.0, *P* < 0.0001). Varroa mite
256 presence, however, showed no association with bald brood (63.5% of colonies with bald brood
257 infested, 71.6% of colonies with only capped brood infested; McNemar's test: χ^2 = 0.7, df = 1, *P*
258 = 0.41).
259 **Within-colony Mite Distributions**
260 We found no significant infestation difference in different frames from the same colony
261 (Wilcoxon signed-rank test: n = 140 colonies, W = 306.5, *P* = 0.18). Approximately equal
262 Tropilaelaps mite abundances in pre-pupal (mean ± se = 5.6% ± 0.7% of cells examined had
263 mites, n = 5,497 cells examined) and pupal (4.6% ± 0.7%, n = 17,808 cells; n = 190 colonies, W
264 = 548.5, *P* = 0.12) worker brood cells further supports a random distribution of Tropilaelaps
265 mites in worker brood. Drone brood had lower Tropilaelaps mite infestation abundance (3.5% ±
266 0.9%, n = 1,474 cells) than worker brood (5.7% ± 0.6%, n = 22,082 cells; n = 98 colonies, W =
267 -616.5, *P* < 0.0001). The opposite was true for Varroa mite infestations, with worker brood
268 having significantly lower Varroa mite infestation abundance (0.4% ± 0.1%) than drone brood
269 (5.0% ± 1.4%; n = 98 colonies, W = 300.0, *P* < 0.0001).
270
271

272 **Tropilaelaps-Varroa Mite Co-infestation**

273 The presence or absence of Tropilaelaps mites in a colony was independent of whether
274 Varroa mites were present (McNemar's test: $\chi^2 = 3.5$, df = 1, $P = 0.06$). However, mite
275 abundances in brood cells suggest an interaction between mite species within a colony. Since the
276 rate of Tropilaelaps mite infestation in worker brood was 4.6% infested cells per colony and the
277 rate of Varroa mite infestation was 0.7%, the expected rate of dual infestation is 0.032%. This is
278 lower than the actual co-infestation rate of 0.1% (Wilcoxon signed-rank test: n = 230 colonies,
279 W = -10,038.0, $P < 0.0001$). This apparent interaction, with dual infections occurring more often
280 then chance would suggest, was also evident in drone brood, where the expected rate of co-
281 infestation was 0.2% and the actual rate was 0.3% (n = 96 colonies, W = -2,088.5, $P < 0.0001$).

282 **Screening Guidelines**

283 Test sensitivities increased markedly at higher Tropilaelaps mite abundances in colonies
284 (Table 3). At the highest infestation abundance level found in our study apiaries, the Bump test
285 detected almost 80% of infected colonies. Thus, larger infestations are more likely to be detected
286 during screening. Figure 4a shows the probability of detecting Tropilaelaps mites as a function of
287 sampling intensity in apiaries with different infestation levels, using the most conservative
288 bumping sensitivity value (0.36; Table 1). With universal infestation, the likelihood of detecting
289 mites increases from 36% when one colony is tested to close to 99% when 10 colonies are
290 inspected.

291 Given the desire to detect a mite infestation in an apiary with 95% confidence, and
292 assuming universal infestation, we calculated that at least seven colonies per apiary should be
293 sampled (Fig. 4a). At our measured 75% Tropilaelaps mite prevalence (Table 1, all tests
294 combined) the sample size necessary to detect infestation is 10 colonies per apiary.

295 Testing seven colonies per apiary and assuming universal infestation within an apiary, we
296 determined the number of apiaries that must be sampled at the regional level to detect an
297 incipient invasion (Fig. 4b). When 1% of apiaries are infested, 312 randomly selected apiaries
298 must be sampled. 5% prevalence in the region drops the sample size to 62 apiaries. Once an
299 infestation reaches 10% prevalence, applying the Bump test to seven colonies in each of 30
300 apiaries is 95% certain to detect the invasion. These numbers assume sampling of seven colonies
301 per apiary. However, current USDA-APHIS protocols, which were designed to quantify Varroa
302 mite load, sample eight colonies (Lee et al. 2010). Sampling eight colonies per apiary with the
303 Bump method, we can be 97.2% confident of detecting at least one Tropilaelaps-infested colony
304 per apiary. Under these protocols, regional sampling should test 307, 61 or 30 apiaries to detect
305 an incipient invasion at 1%, 5% or 10% infestation prevalence, respectively.
306
307 **Discussion**
308 This study was initiated to determine a rapid method for detecting Tropilaelaps mites
309 with 95% confidence. Early invasion detection by this damaging and rapidly spreading honey
310 bee pest is critical to preventing further honey bee loses and a shortage of these vital crop
311 pollinators. Our results support the Bump test as the best simple method to test for Tropilaelaps
312 mite presence during apiary surveys. This method involved bumping the frame over a metal pan
313 and counting the mites that fell. It had a sensitivity of 50.0% for colonies in which Tropilaelaps
314 mite infestation was verified, and 36.3% when universal infestation was assumed. The sensitivity
315 of this test was even greater (79.3%) for colonies at or just below the 5% Tropilaelaps mite
316 infestation level seen in our acaricide-treated colonies.

While daily mite drop, examining worker brood and re-bumping after examining brood were more sensitive than the Bump test, each has associated problems that make them less feasible for large surveys. Concern over differentiating this mite from natural hive debris, the time required to carefully separate the two (Ostiguy and Sammataro 2000) and the need to return to hives 24 hours after sticky board insertion made the Drop test impractical for large-scale screening. Both the Worker Brood and Post-Bump test required exposing brood for extended periods of time, thus increasing brood mortality during the examination. In particular, the Post-Bump method destroyed brood, which beekeepers may object to. This method also takes longer than the Bump test since brood cells are uncapped before bumping. The Post-Bump test could potentially be used without removing the brood, and could be one means to increase the sensitivity of the bump method.

Our results roughly agree with previously published Tropilaelaps mite prevalence and abundance data from other Asian countries. Prevalence of infested hives was 86.3% in Chinese European honey bee hives in Autumn (Luo et al. 2011) and 76.5% in giant honey bee hives from northern Thailand (Burgett et al. 1990). Tropilaelaps mite abundance in worker brood cells in our study (4.6%) was slightly lower than in Pakistani European honey bee colonies (8.1%, Waghchoure-Camphor and Martin 2009) but noticeably higher than in giant honey bee hives (1.8%, Burgett et al. 1990). The difference between European and giant honey bees may be due to species-specific responses to infested cells. European honey bee workers typically open cells and remove diseased brood from the hive, while giant honey bee workers leave sealed infested cells, which prevents adult mites from departing to lay their own eggs (Woyke et al. 2004).

The bald brood condition was a fairly reliable indicator of Tropilaelaps mite infestation, but should not be used as the sole diagnostic method. Multiple hive pests cause bald brood,

340 including Varroa mites and wax moth larvae (Villegas and Villa 2006). Higher Tropilaelaps mite
341 abundances in bald than capped cells, however, suggest that bumping will have higher sensitivity
342 with frames that have at least one uncapped cell. In addition, increasing bald brood rates within a
343 hive or apiary should warn beekeepers to test for Tropilaelaps mites.

344 The only within-colony mite distribution pattern we found was greater mite abundance
345 and prevalence in worker than drone brood cells. This counters information currently provided to
346 beekeepers (e.g. DEFRA 2005, Ritter 2008), which is based on a report that gives only ranges
347 (Burgett et al. 1983). Given this discrepancy, and the equal mite abundances in drone and worker
348 cells found by Waghchoure and Martin (2009), the question of whether Tropilaelaps mite
349 females prefer to lay eggs in worker or drone brood cells must be re-examined.

350 We assumed that all hives are Tropilaelaps-positive in any apiary in which one hive tests
351 positive for Tropilaelaps mites. Mite biology and high measured mite prevalence support this
352 assumption. Managed European honey bee colonies in the Tropilaelaps mite's native range
353 almost always become infested without mite control (Ritter and Akratanakul 2006), indicating
354 frequent mite movement between hives. Adult mites can leave the nest on foraging honey bees,
355 and transfer to bees from a different nest during frequent interactions between honey bee
356 colonies (Evans and Schwarz 2011). Indeed, simultaneous or sequential floral visitation, and
357 robbing materials from neighboring nests are becoming recognized as important bee pathogen
358 transmission routes (e.g., Durrer and Schmidhempel 1994, Lindstrom et al. 2008).

359 Based on the most conservative Bump test sensitivity data, we suggest the following
360 surveillance protocol for early detection of a Tropilaelaps mite invasion with 95% confidence.
361 First, regional or national surveys should examine at least seven colonies per apiary, bumping
362 one frame per hive as described in the USDA's Protocol for National Honey Bee Disease Survey

363 (USDA APHIS 2012). Current U.S. sampling protocols examine eight colonies per apiary,
364 meeting our recommendation. This calculation assumes universal infestation within an infested
365 apiary. Our assumption is valid not only for the biological reasons discussed above, but also
366 because colonies in a newly-invaded region will not be undergoing treatment for Tropilaelaps
367 mites. Current Varroa mite treatments are applied two to three times per year, much less
368 frequently than the every two weeks necessary to control Tropilaelaps mites. Second, we
369 recommend that surveys test 312 (or 307 if examining eight colonies per apiary) apiaries within a
370 region for early invasion detection. The International Office of Epizootics (OIE) recommends
371 that screening protocols have a 95% probability of detecting a 1% infestation in a region (OIE
372 2012). Invasions still at very low regional prevalence can potentially be controlled or eliminated
373 via quarantines, delimiting surveys and aggressive destruction of all infested hives. Our results
374 provide the information necessary for the sufficient and efficient Tropilaelaps mite monitoring
375 necessary to prevent the increased economic and agricultural losses that would result from the
376 introduction of Tropilaelaps mites outside their native range.
377

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Table 1: **Mite detection and sensitivity of six detection techniques**

					Test sensitivity	
Detection test	**Number of colonies sampled (# apiaries)**	**Mite prevalence (%)**	**Mite abundance (mean ± SE)**	**Number of sampled colonies found mite-positive by at least one test**	**Infestation verified**	**Universal infestation assumed**
***Tropilaelaps* mite**						
Drop	96 (3)	53.0 ± 13.2	5.4 ± 1.9[a]	64	81.3	54.2
Wash	96 (3)	4.8 ± 2.7	5.7 ± 2.9[b]	64	7.8	5.2
Bump	201 (10)	35.3 ± 8.7	1.5 ± 0.28[c]	146	50.0	36.3
Worker Brood	231 (10)	58.6 ± 7.5	4.6 ± 0.06[d]	173	75.7	56.7
Drone Brood	97 (9)	13.0 ± 3.8	3.5 ± 0.09[d]	75	32.0	24.7
Post-Bump	169 (10)	40.9 ± 10.1	3.9 ± 0.7[c]	135	61.5	49.1
All tests combined	236 (10)	75.9 ± 5.3	N/A	236	N/A	74.6
***Varroa* mite**						
Drop	96 (3)	75.7 ± 6.6	1.6 ± 0.16[a]	75	94.7	74.0
Wash	96 (3)	18.9 ± 15.6	19.9 ± 4.6[b]	75	24.0	18.8
Bump	201 (10)	6.6 ± 1.7	0.2 ± 0.15[c]	126	10.3	6.5
Worker Brood	231 (10)	27.8 ± 6.4	0.7 ± 0.01[d]	152	40.1	26.4

Detection test	Number of colonies sampled (# apiaries)	Mite prevalence (%)	Mite abundance (mean ± SE)	Number of sampled colonies found mite-positive by at least one test	Infestation verified	Universal infestation assumed
Drone Brood	97 (9)	16.1 ± 4.2	5.0 ± 1.43[d]	63	47.6	30.9
Post-Bump	170 (10)	23.3 ± 6.6	0.9 ± 0.18[c]	105	44.8	27.6
All tests combined	236 (10)	64.8 ± 5.8	N/A	236	N/A	66.5

Prevalence indicates the mean ± SE percentage of colonies with mites in each apiary. One apiary did not have any drone brood in examined colonies.

[a]Mites dropped per colony in 24h

[b]Mites per 100 bees

[c]Mites removed from one frame per colony

[d]Cells infested per 100 (worker) or 20 (drone) cells examined

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Table 2: Time investment required for each detection test

	Per colony				Per apiary (8 colonies)	
Diagnostic test	**Preparation time (min)**	**Implementation time (min)**	**Analysis time (min)**	**Total time (min)**	**Testing time (h)**	**# trips required**
Drop	10	4	15	29	3.87	2
Wash	5	5	5 – 10	15 – 20	2 – 2.67	1
Bump	5	5	5	15	2	1
Worker brood	5	15		20	2.67	1
Drone Brood	5	5		10	1.33	1
Post-Bump	5	7	5	17	2.27	1

Preparation includes opening hives and removing frames. Implementation refers to all procedures carried out in the apiary once frames are removed. Most analysis occurred in the lab, with samples brought back from apiaries. Bump and Post-Bump counts, and worker and drone brood examinations, occurred in the field.

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Table 3: Tropilaelaps mite detection test sensitivities at minimal, medium and high infestation abundances in worker brood

	Worker brood infestation level		
Detection test	**0%**	**0.1-4.5%**	**>4.5%**
Drop	41.7	66.7	91.7
Wash	3.3	8.3	8.3
Bump	11.2	35.6	79.3
Drone Brood	3.3	22.6	47.1
Post-Bump	16.1	49.1	87.0

Test sensitivity was calculated assuming universal infestation of all hives in an infested apiary (standard B, see text for details). We did not calculate sensitivities of the Worker Brood test because this test was the source of infestation level categorization.

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Figure Legends

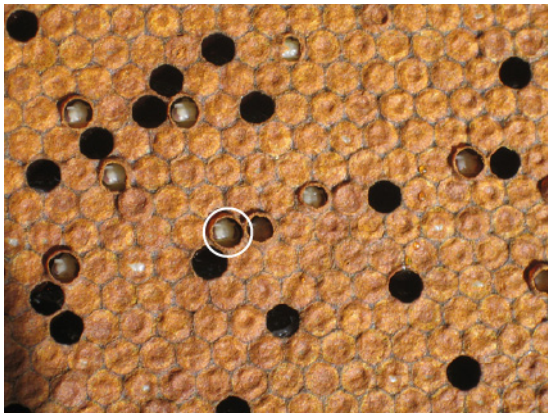
Fig. 1: "Bald brood" (circled), a condition where the capping over pupal bees has been removed, is thought by beekeepers to indicate heavy Tropilaelaps mite infection. Photo credit J. Pettis, USDA-ARS.

Fig. 2: Size comparison of Varroa (left) and Tropilaelaps (right) mites. Photo credit I.B. Smith, USDA-ARS.

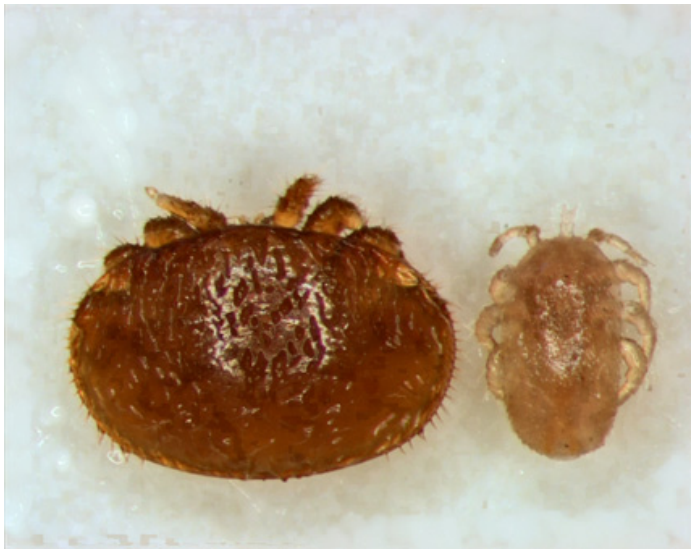
Fig. 3: Material collected on sticky boards over 24 h. Mites and hive debris are difficult to rapidly distinguish. Photo credit J. Pettis, USDA-ARS.

Fig. 4: a) Probability of detecting Tropilaelaps mites when different numbers of colonies are sampled, at various apiary-wide mite infestation rates. The sensitivity of the test here as set at 36%. b) Probability of detecting a recent Tropilaelaps mite invasion when different numbers of apiaries are sampled across a region, at various mite prevalence values. Vertical lines show where each curve reaches 95% detection.

Fig. 1



498 Fig. 2



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501 Fig. 3

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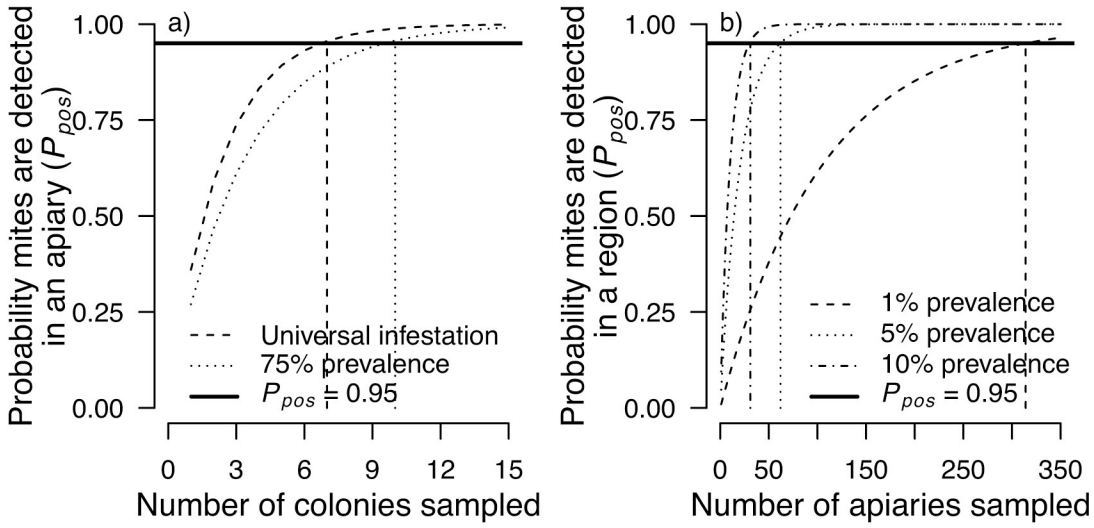
504 Fig. 4


a)
b)
Probability mites are detected in an apiary (P_{pos})
Probability mites are detected in a region (P_{pos})
1.00
0.75
0.50
0.25
0.00
Universal infestation
75% prevalence
P_{pos} = 0.95
1% prevalence
5% prevalence
10% prevalence
0 3 6 9 12 15
Number of colonies sampled
0 50 150 250 350
Number of apiaries sampled

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