BRANCHED AMPLIPHILIC PEPTIDE CAPSULES TOXICITY STUDIES IN APIS MELIFERA

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Introduction:

This report outlines two separate toxicity studies conducted on *Apis melifera*. The first study follows the parameters of the EPA Litmus test and the second study delves deeper into possible BAPC toxicity surpassing the standards of the EPA definitive test. Each study was performed under EPA guidelines including harvesting location, race, containment conditions, test duration, age, acclimation, diet and feeding solutions, and care/handling.

For both studies, *Apis melifera (honey bees)* were collected from one of eight locally maintained hives, from the same geographical location (GPS 39° 17′ 8.03713″ N 99° 32′ 54.76309″ W) and were subjected to Branched Amphiphilic Peptide Capsules (BAPCs) to establish toxicity values. BAPCs were obtained from Phoreus Biotech lyophilized and were reconstituted with MilliQ water at a concentration of 50 µM. Bees were stored in bee buses utilized for transport and feeding studies. All bee studies were completed at Fort Hays State University in an insect incubator with hive lighting conditions and environmental parameters set at 32^{o}C and 50-60% relative humidity for the duration of the studies mimicking normal hive conditions. Prior to beginning the study, bees were allowed to acclimate to the conditions for 24 hours although this parameter was not required per EPA guidelines.

Study 1: EPA Litmus Test

According to EPA guidelines for a Limit test, for test substances expected to have relatively low toxicity, as in the case for BAPC's in place of a definitive test a limit test may be conducted with a single contact dose level, the limit dose, plus a control group. In these situations, it is only necessary to ascertain that the 48-hr LD50 is above the limit dose (i.e., 48-h LD50 > limit dose). In a honey bee acute contact limit tests at least 25 bees are exposed to the "limit dose" with the same number of bees in appropriate controls. For most pesticides the limit dose is 25 µg of active ingredient per bee (25 µg a.i./bee). BAPCs were utilized at these concentrations for four trials including a control. These bees were subjected to a diet with BAPCs in the standard honey bee diet consisting of a 50% weight/volume (w/v) solution of sugar/water (500 grams/liter) provided *ad libitum* throughout the holding and test periods.
Distilled MilliQ water was used for the sugar diet solutions. Following test guidelines, 25 bees were used, and the test was conducted for 48hrs.

At test termination (48 hours), since one or fewer bees were dead at the limit dose, the acute contact LD50 is considered to be greater than the limit dose (i.e., LD50 > limit dose). This is because the Binomial Theorem predicts that when 25 bees are tested, the probability of seeing <1 dead bee if the true 48-h LD50 is at or below the limit dose is <0.001. Conversely the probability of seeing 2 or more dead bees if the true 48-h LD50 is at or below the limit dose is >0.999. Therefore, if <1 mortality occurs among the 25 bees tested, the 48-h LD50 is reported as greater than the limit dose (i.e., 48-h LD50 >25 µg/bee for pesticides). Data (table 1.) shows that following the Litmus Test, BAPCs pose no significant mortality to honey bees.

Study 2: Enhanced Long Duration Tests

For this test, I wanted to evaluate long term exposure to BAPCs as it pertains to my research goals and is vital information for future applications of BAPCs by Phoreus. It is important to note that although bees in the wild can and will persist and live for 6 to 9 months, in a laboratory setting the lifespan is near the two week mark typically around 16-18 days.

Housed within the incubator, containment vessels fed bees consisting of a sucrose solution mixed with BAPCs at concentrations of 50µM, 5µM, 500nM, 50nM, 50pM, 5pM and a control with no BAPCs. Each trial consisted of 400 bees and was simultaneously conducted at all BAPC concentrations. Daily counts of bee deaths were utilized to establish death curves to allow for statistical comparison (see figure 1.)

Although increased BAPC concentration initially appears at first glance to cause an increased death of bees, the statistical results indicated by the hazard rates in the log-rank comparison tests show that they actually are no different than that of the controls. The log- rank test, utilized at a confidence interval of 99% showed that the highest concentration tested, 50µM is statistically insignificant and therefore all others are also insignificant. Parameters of each Log-rank test is listed in Table 2.

Based on previous literature of aphid feeding studies, a commercial dosage of BAPCs in application would be in the nano to pico molar ranges. Environmentally, I can state with that information in mind, it is obvious that BAPCs pose no threat to honey bees at the exposure ranges that would be seen in the environment if utilized in a similar commercial application. Moreover, as a typical commercial application would be a one-time application spanning a few days, versus a continuous application as seen in these studies, I would be unsurprised if those few deaths associated at the 50µ and 5µM concentrations would fall off and mirror the control studies. Unequivocally these results show that BAPCs used at commercial concentrations pose no threat to one of the most significant pollinator species seen in the world.

Conclusion:

These two studies show statistically that for both the short term and long term exposure to BAPCs, there is no significant death to honey bees. Therefore, I would corroborate that BAPCs could be considered as an inert delivery vector for the treatment of bees, and they do not pose a significant threat to bee populations in the commercial and noncommercial levels utilized in the aforementioned trials.

Tables and Figures:

BAPC Litmus Test: Live bees					
N=25	Control	Trial 1	Trial 2	Trial 3	Trial 4
12hrs	25	25	25	25	25
24hrs	25	24	24	24	25
36hrs	24	24	24	24	24
48hrs	24	24	24	24	23

Table 1. Litmus Test toxicity studies.

Log-rank test Results @99% Confidence Interval			
Concentration	z value	p value	Result
50µM	1.870	0.061	No significant difference
5µM	1.060	0.290	No significant difference
500nM	0.570	0.570	No significant difference
50nM	0.015	0.990	No significant difference
50pM	0.034	0.970	No significant difference
5pM	0.210	0.840	No significant difference

Table 2. Bee cytotoxicity Log-rank test statistical data.

Control			
n=400	Live	Dead	percent
Day 1	400	0	0.0
Day 2	380	20	5.0
Day 3	339	61	15.3
Day 4	329	71	17.8
Day 5	280	120	30.0
Day 6	251	149	37.3
Day 7	222	178	44.5
Day 8	198	202	50.5
Day 9	163	237	59.3
Day 10	137	263	65.8
Day 11	82	318	79.5
Day 12	50	350	87.5
Day 13	18	382	95.5
Day 14	17	383	95.8
Day 15	9	391	97.8
Day 16	0	400	100.0

Table 3. Bee cytotoxicity control. No BAPC present in diet.

50uM			
n=400	Live	Dead	percent
Day 1	400	0	0.0
Day 2	359	41	10.3
Day 3	289	111	27.8
Day 4	271	129	32.3
Day 5	250	150	37.5
Day 6	187	213	53.3
Day 7	117	283	70.8
Day 8	75	325	81.3
Day 9	26	374	93.5
Day 10	3	397	99.3
Day 11	1	399	99.8
Day 12	0	400	100.0
Day 13	0	400	100.0
Day 14	0	400	100.0
Day 15	0	400	100.0
Day 16	0	400	100.0

Table 4. Bee cytotoxicity test. 50µM BAPC present in diet.

5uM			
n=400	Live	Dead	percent
Day 1	400	0	0.0
Day 2	365	35	8.8
Day 3	293	107	26.8
Day 4	288	112	28.0
Day 5	261	139	34.8
Day 6	212	188	47.0
Day 7	180	220	55.0
Day 8	131	269	67.3
Day 9	91	309	77.3
Day 10	69	331	82.8
Day 11	35	365	91.3
Day 12	11	389	97.3
Day 13	3	397	99.3
Day 14	0	400	100.0
Day 15	0	400	100.0
Day 16	0	400	100.0

Table 5. Bee cytotoxicity test. 5µM BAPC present in diet.

500nM			
n=400	Live	Dead	percent
Day 1	400	0	0.0
Day 2	374	26	6.5
Day 3	313	87	21.8
Day 4	304	96	24.0
Day 5	269	131	32.8
Day 6	242	158	39.5
Day 7	200	200	50.0
Day 8	175	225	56.3
Day 9	144	256	64.0
Day 10	113	287	71.7
Day 11	52	348	87.0
Day 12	32	368	92.0
Day 13	15	385	96.3
Day 14	1	399	99.8
Day 15	0	400	100.0
Day 16	0	400	100.0

Table 6. Bee cytotoxicity test. 500nM BAPC present in diet.

50nM			
n=400	Live	Dead	percent
Day 1	400	0	0.0
Day 2	375	25	6.3
Day 3	322	78	19.5
Day 4	309	91	22.8
Day 5	275	125	31.3
Day 6	255	145	36.3
Day 7	211	189	47.3
Day 8	187	213	53.3
Day 9	149	251	62.8
Day 10	123	277	69.3
Day 11	77	323	80.8
Day 12	58	342	85.5
Day 13	29	371	92.8
Day 14	20	380	95.0
Day 15	4	396	99.0
Day 16	0	400	100.0

Table 7. Bee cytotoxicity test. 50nM BAPC present in diet.

50pM			
n=400	Live	Dead	percent
Day 1	400	0	0.0
Day 2	384	16	4.0
Day 3	340	60	15.0
Day 4	315	85	21.3
Day 5	290	110	27.5
Day 6	260	140	35.0
Day 7	228	172	43.0
Day 8	194	206	51.5
Day 9	163	237	59.3
Day 10	140	260	65.0
Day 11	75	325	81.3
Day 12	53	347	86.8
Day 13	21	379	94.8
Day 14	15	385	96.3
Day 15	6	394	98.5
Day 16	0	400	100.0

Table 8. Bee cytotoxicity test. 50pM BAPC present in diet.

5pM			
n=400	Live	Dead	percent
Day 1	400	0	0.0
Day 2	381	19	4.8
Day 3	333	67	16.8
Day 4	328	72	18.0
Day 5	279	121	30.3
Day 6	250	150	37.5
Day 7	221	179	44.8
Day 8	199	201	50.3
Day 9	163	237	59.3
Day 10	137	263	65.8
Day 11	81	319	79.8
Day 12	48	352	88.0
Day 13	17	383	95.8
Day 14	16	384	96.0
Day 15	7	393	98.3
Day 16	0	400	100.0

Table 9. Bee cytotoxicity test. 5pM BAPC present in diet.

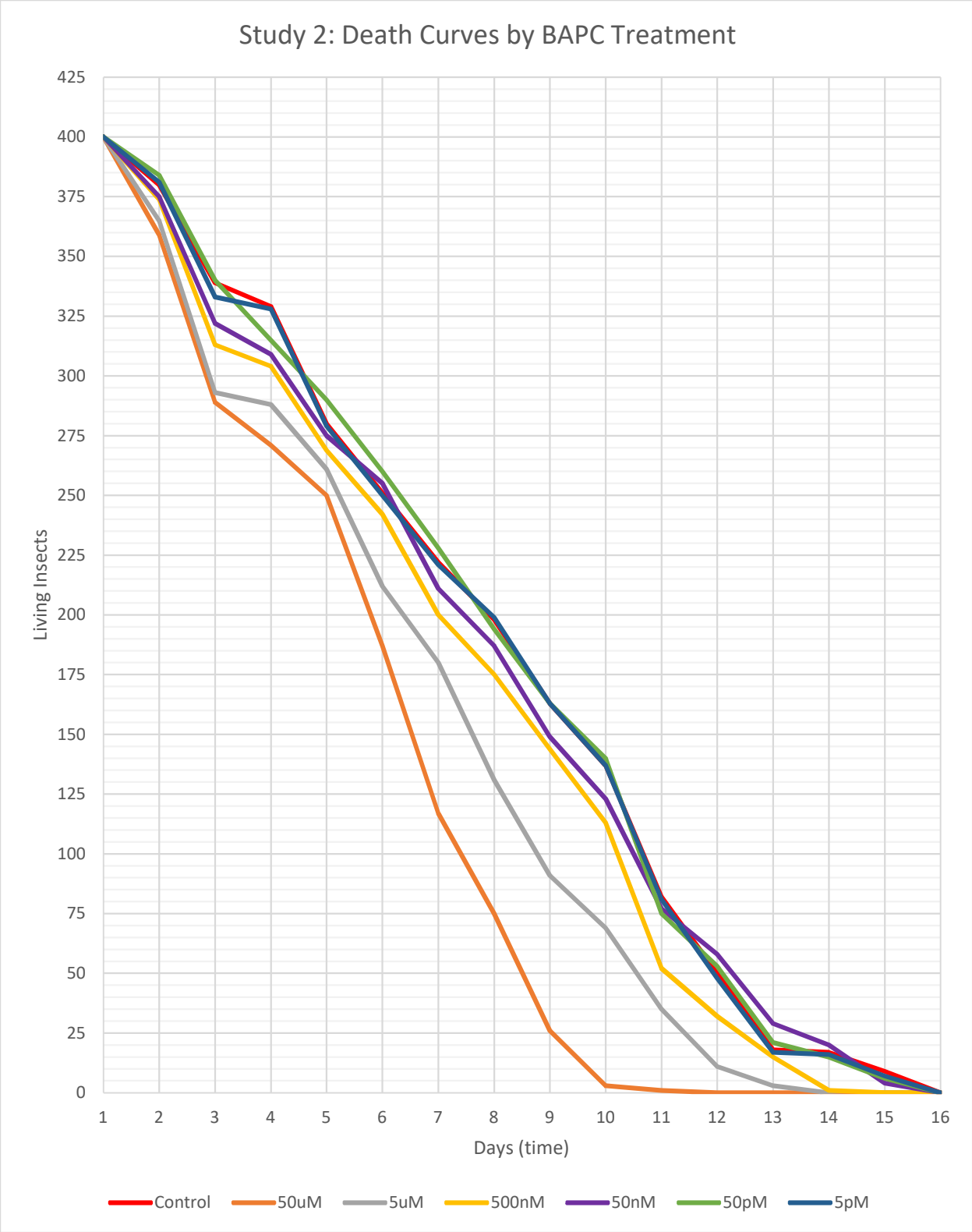

Figure 1. Bee cytotoxicity death curves by various BAPC treatment present in diet.