Sub-lethal effects of pesticide residues in brood comb on worker honey bees

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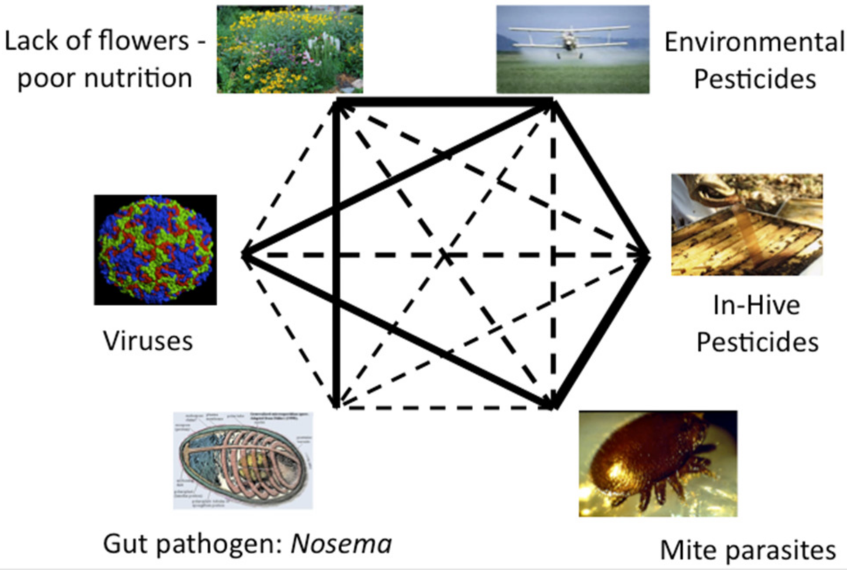
Why are colonies dying? A puzzle of interactions



Research objectives

Study 1: Pesticide load survey

- Which, how many, & what levels are pesticide residues found in brood comb?
- Correlation between failing colonies & pesticide load?

Study 2: Brood effects & adult longevity

- Development & survivability (egg to adult emergence)
- Adult lifespan

Study 3: Susceptibility expt.

-To other pests and pathogens

Translating pesticide jargon

Hard treatments: synthetic or inorganic compounds for Varroa control

Pesticide class	Active ingredient	Brand name
pyrethroid	Tau-fluvalinate	Apistan, Hivestan
organophosphate	Coumaphos	Checkmite+
formamidine	Amitraz	Apivar, Tactic

Study 1: Pesticide load survey

Source of "dirty combs"

5 categories of samples (n_{total}=98):

1) PNW migratory colonies (poor) (n=24)
2) CRC (collaborative research colonies) (n=24)
3) QPO queen-producers (n=19)
4) FWS commercial foundation wax (n=7)
5) CCD suspect "CCD" (Dr. Jeff Pettis USDA, MD) (n=24)

Chemical analysis:

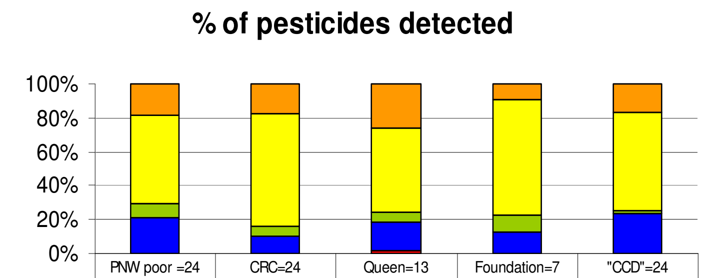
Samples sent to Roger Simonds at the USDA-AMS lab in Raleigh N.C.

171 most commonly used pesticides tested (including coumaphos, fluvalinate, & amitraz metabolite (DMPF)).

Residues in combs determined with GC/MS.

What did we find?

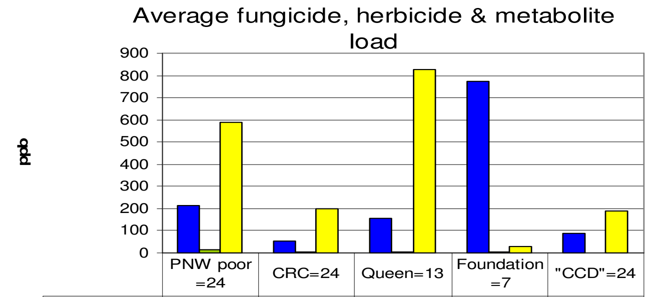
Out of 171 screened, we found **66** different pesticides, **10** metabolites, and **1** adjuvant (piperonyl butoxide)



	PNW poor =24	CRC=24	Queen=13	Foundation=7	"CCD"=24
metabolites	19%	17%	26%	9%	17%
Insect/Acaricide	51%	67%	50%	68%	58%
Herbicide	9%	6%	7%	9%	2%
Fungicide	21%	10%	16%	13%	23%
adjuvants	0%	0%	2%	0%	0%

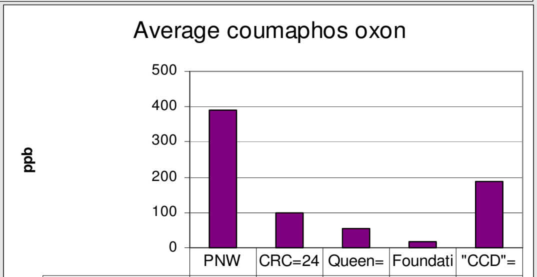
Of the 47 insect/acaricides, 36 (77%) are toxic to bees!!!

Fungicides:
- -detected in all categories
- -highest in foundation wax



	PNW poor =24	CRC=24	Queen=13	Foundation =7	"CCD"=24
Fungicide	212	55	156	773	86
Herbicide	16	3	3	5	0
metabolites	590	200	827	28	192

Metabolites:
- -break down products
- - coumaphos oxon is a major metabolite found in each category



	PNW	CRC=24	Queen=	Foundati	"CCD"=
coumaphos oxon	390	99	56	17	188

Commercial Foundation Wax

- Recycled cell caps (used to store honey in comb)
- Used by beekeepers to draw new comb
- Wax caps removed (during honey extraction) & processed by boiling
- Boiling comb: no effect on residue levels in contaminated comb
- Heated, melted, & processed comb with known amounts of pesticides revealed residue levels 1.7x higher after processing

(*Bogdanov et al. 1996*)

Commercial Foundation Wax

High levels of 7 different fungicides in FWS, support prevalence of pesticides brought back by forager bees.

In addition, 21 insecticides, 4 herbicides & 4 metabolites were also detected.

Fungicides may affect bees

Direct effects:

- Increased mortality & growth abnormalities in larvae when fed fungicides

- Delayed effects – (captan), after 7 days toxicity of captan was 3-4x more toxic than assessment 72hrs after the same single dose exposure *(Mussen et al. 2004)*

Indirect effects:

- Synergistic effects (**10-100x**) (honey bees) when EBI fungicides are combined with pyrethroids

  (Pilling and Jepson 1993)

  EBI fungicides/neonicotinoids

  non EBI fungicides/neonicotinoids (shorter period)

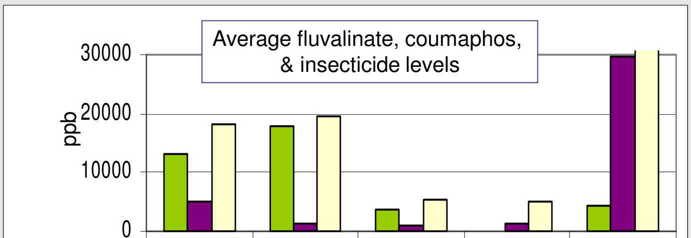
  (Schmuck et al. 2003)

- Reduced repellency of pyrethroid/fungicide mixtures

  (Thompson and Wilkins 2003)

What insecticides did we find?

In-hive miticides account for majority of the insecticide load



	PNW poor =24	*CRC=24	Queen=13	Foundation=7	"CCD"=24
fluvalinate	13086	17907	3565	0	4428
coumaphos	4970	1407	1133	1243	29730
Insect/Acaricide	18130	19479	5475	4890	34172

*After one year, 50% of CRC were dead-outs!

Fluvalinate

fluvalinate	PNW poor	CRC	Queen	Foundation	"CCD"
average	4214	17907	3565	3357	4428
range	164-**12000**	**4010-92600**	127-**16400**	236-**12500**	646-**21300**

<u>Sub-lethal effects of fluvalinate in wax on queen rearing:</u>

Significant reduction of queen weight at fluvalinate level of 3,550 ppb.
(Haarmann, T. et al. 2002. .*J. Econ. Entomol.* 95 (1): 28-35)

83 (97%) samples had fluvalinate and 56 (67%) had fluvalinate levels that exceed 3,550 ppb!

Note: the tolerance level of fluvalinate in honey is 50ppb!

Coumaphos

coumaphos	PNW poor	CRC	Queen	Foundation	"CCD"
average	4190	1407	1133	1243	29730
range	205-22100	733-4130	0-4440	63.5-3140	2290-**226000**

Sub-lethal effects of coumaphos in wax on queen rearing:

50% rejection of queen cells at coumaphos level of 100,000ppb.
(Pettis, J.S. et al. 2004. *Apidologie* 35: 605-610)

Significant reduction in queen & ovary weight at coumaphos level of 50,000 ppb. (Haarmann, T. et al. 2002. .*J. Econ. Entomol.* 95 (1): 28-35)

Coumaphos was present in 85 (98.8%) of samples!

Note: the tolerance level of coumaphos in honey is 100ppb!

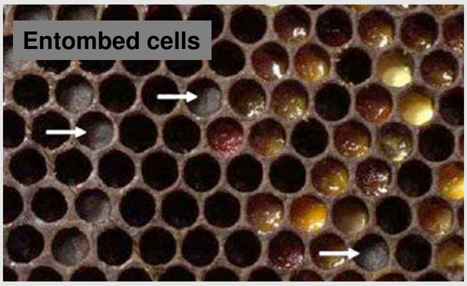
Contamination in comb

Residues from "in-hive" and environmental pesticides **remain** and **accumulate** in the comb for years

(Mullin, C.A. et al. 2010. *PLoS one*. 5(3): e9754) – study found **121** different pesticides & metabolites within 887 comb, pollen, and bee samples.

-60% of comb had at least one systemic pesticide

-over 47% had fluvalinate, coumaphos, & chlorothalonil



In-hive pesticide concerns

- Additive effect: two substances in combination produce a total effect the same as the sum of the individual effects (1+1=2)
  - *Ex: similar pesticides, multiple applications*
- Synergistic effect: the interaction of multiple compounds so that the sum of the combined effect is greater than either individual effect. (1+1>2)
  - *Ex: Apistan (pyrethroid) + Checkmite (organophosphate)*

Synergy of in-hive treatments

(Johnson, R.M et al. 2009. *J. Econ. Entomol.* 102(2): pp 474 – 479)

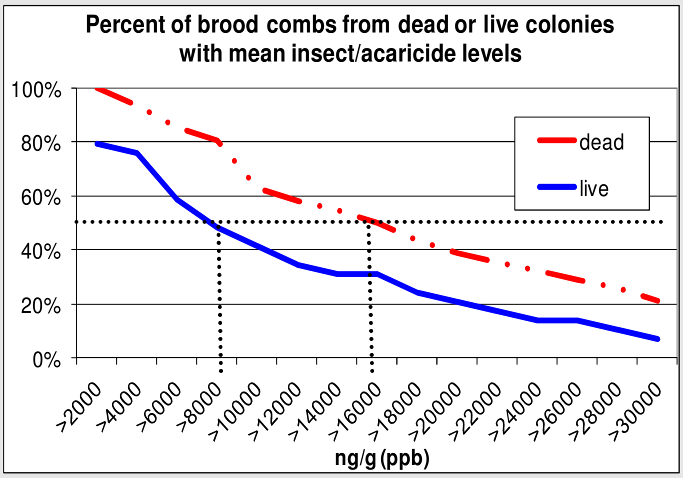
Pretreatment	x-fold synergy
0.3 µg coumaphos	2.1x (↑ fluvalinate toxicity)
3.0 µg coumaphos	4.4x (↑ fluvalinate toxicity)
10.0 µg coumaphos	32.1x (↑ fluvalinate toxicity)
1-3 µg fluvalinate	3.4x (↑ coumaphos toxicity)

Synergy with environmental pesticides:

- pyrethroid & piperonyl butoxide (PBO)= 980X;
- ergosterol biosynthesis inhibitors (EBI) fungicides & insecticides =10-100X;

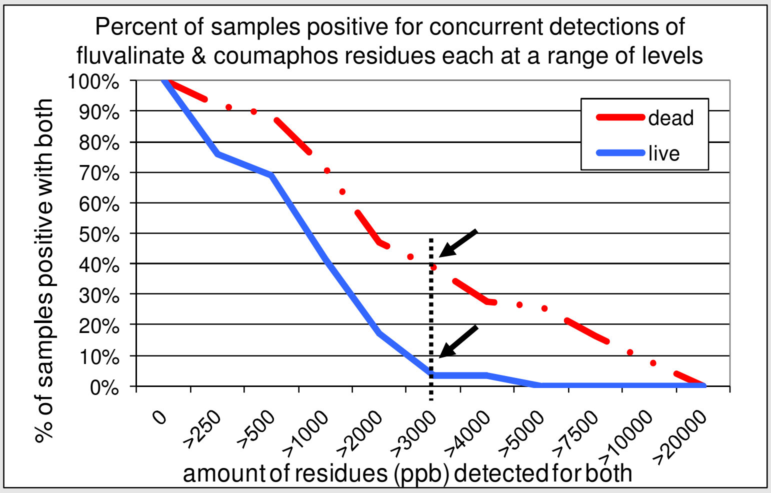
Average insecticide level was significantly higher in brood comb from dead colonies (p=0.034).

Brood comb from dead colonies had 2x the amount of insecticide residues than from live colonies.



Concurrent detections of miticides

34% of brood comb from dead colonies had 3,500 ppb of each compared to 3% of brood comb from live colonies



Graphs are not suggesting Varroa treatments killed colonies

Only that there may be a connection between pesticide load and the health of colonies

There's more to the story…

Study 2: Brood effects study

Wu et al. (2011) PLoS ONE 6(2): e14720.

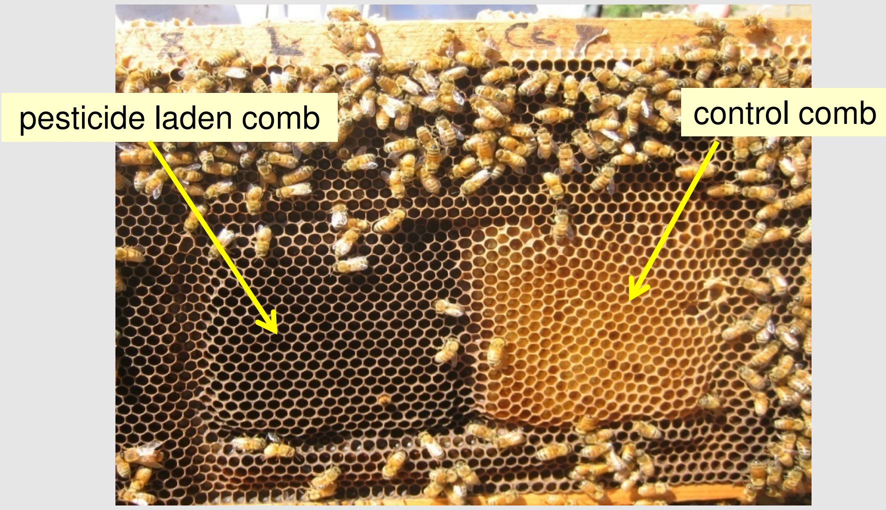
Controls – newly drawn comb (coumaphos 21 ppb) or old comb from feral colonies with no detectable residues.

Treatments – (13 brood combs from previous survey)

- 24 insecticides, 9 fungicides, 3 herbicides, and 5 metabolites = 41 total
- # of pesticides ranged from 4 to 17, averaging 10

Active ingredient	Family	Average (ng/g)	Minimum (ng/g)	Maximum (ng/g)	LOD (ng/g)
Fluvalinate	PYR	6,712	164	24,340	1
Coumaphos	OP	8,079	281	22,100	1
Coumaphos oxon	metabolite	596	10	1,850	5

Study 2: Brood effects & longevity



Enclosed queen laid eggs for 24 hrs on either block
(up to 1000 eggs daily)

Brood effects

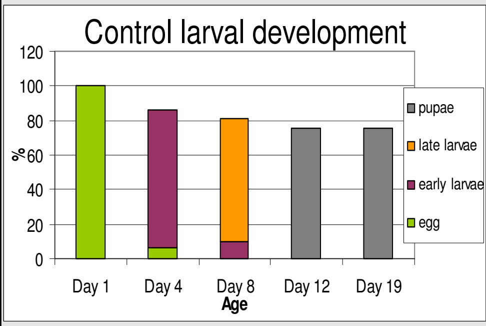
After 24 hrs. development of brood recorded at:

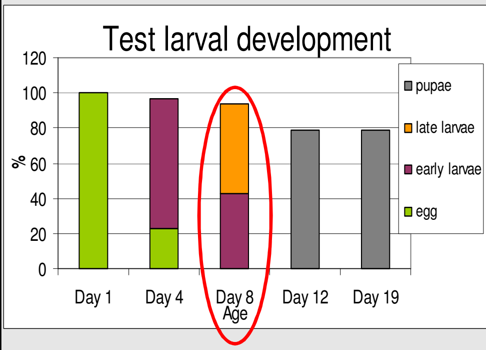
day 1 (egg) — **day 4** (1st instar) — **day 8** (5th instar) — **day 12** (capped pupae) — **day 19** (incubation)

(Capped brood incubated (34°C))

Newly emerged adults

1) cage longevity tests ← → 2) susceptibility expt





Control

Pesticide	ppb
Coumaphos	**20.9**

Test

Pesticides	ppb
3-hydroxycarbofuran	**23**
Aldicarb	**20**
Carbofuran	**32**
Chlorothalonil	4
Clothianidin	**35**
Coumaphos	**22100**
Coumaphos Oxon	**1850**
Cyfluthrin	**7.9**
Dinotefuran	**97**
Diphenylamine	281
Endosulfan 1	**1**
Fluvalinate	**164**
Imidacloprid	**45**
Malathion Oxon	**22**
Oxamyl	**22**
Thiacloprid	**113**
Thiamethoxam	**38**

2 Fungicides

3 Carbamates
+ 1 metabolite

1 OP + 2 metabolite

1 Cyclodiene

2 Pyrethroids

5 Neonicotinoids

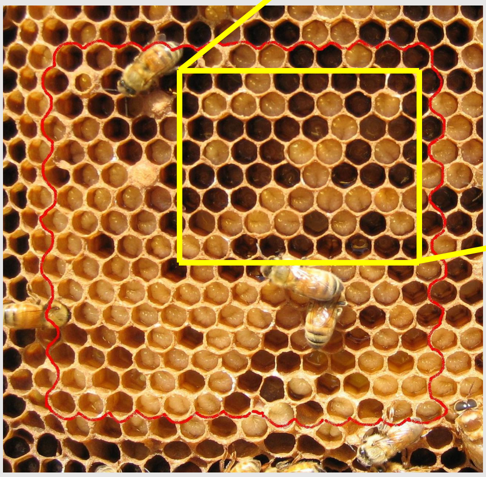
Total = 17

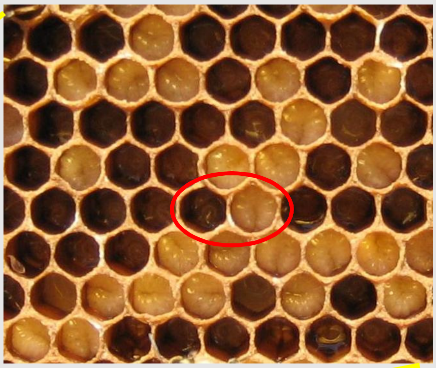
highly toxic

mod. toxic

Treatment comb: day 8

46% of treatment larvae expressed delayed development





Delayed development   Normal development

Delayed larval development

Worker bees from another 3 treatment combs (sampled from CCD colonies) had similar effects.

An average of 19% of eggs remained unhatched at day 4

60-80% of those eggs were removed by day 8

Spotty brood pattern:
reduces brood production efficiency

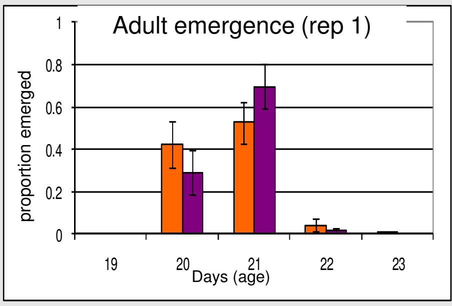
(queens must seek out empty cells, nurse bees must scatter around to care for brood)

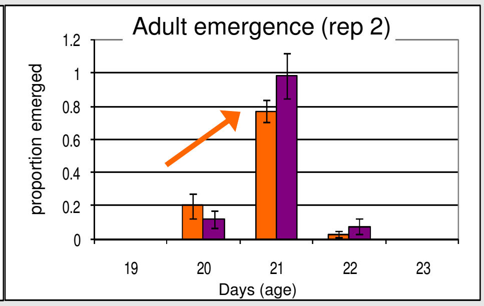
(Mackensen 1950)

Adult worker emergence

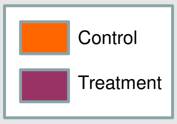
Typical worker honey bee emergence occurs at day 20-21 of development.

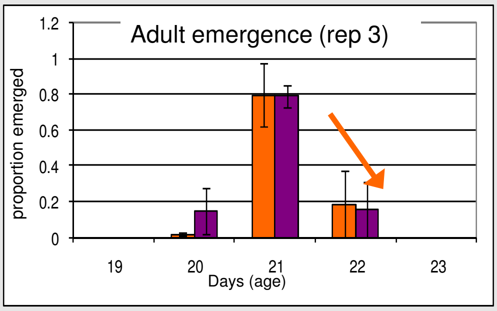
Brood combs used multiple times





Delayed emergence?





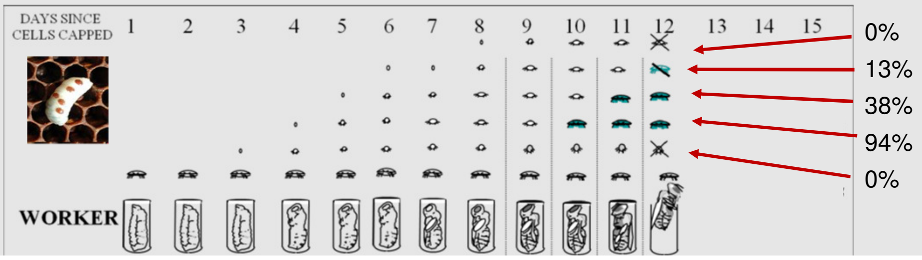
Effects on the colony?

Understanding *Varroa destructor:*

External parasitic mite
Feeds on bee "blood"
Circulates bee ***viruses***
Reason for ***in-hive pesticides***

Varroa development

Pregnant mites leaves cell with adult bee; male & immatures die in cell

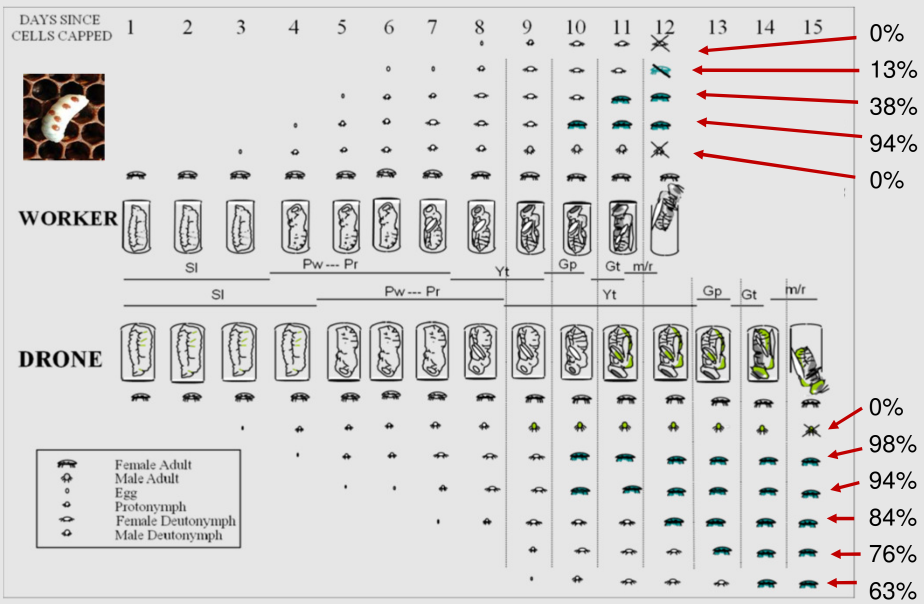


The mite family is completed by 15th day of bee development:

- mother mite & 4 progeny: (1st son, 1st daughter, 2nd daughter, 3rd daughter (egg))
- 3rd daughter only has a 13% chance of reaching adulthood.

http://www.ars.usda.gov/services/docs.htm?docid=2744&page=14

Varroa fecundity in drone brood



Varroa preference for drone brood

Development time for drones is 23-24 days

Extra 2.5 days provides time for an additional 1-2 daughter mites to mature…the average # of daughters per mite in drone cells is about **2-2.5**

Average # of daughters per mite in worker cells is **1.4-1.5**

Average # of daughters per mite in worker cells with bees expressing delayed emergence?

Altered physiology of mite infested worker bees

(Amdam et al. 2004)

" Compared with non-infested workers, adult bees infested as pupae do not fully develop physiological features typical of long-lived wintering bees."

- reduced body weight, hemolymph volume, & abdominal carbohydrates
- reduced levels of key proteins (vitellogenin) for overwintering

Late-autumn treatments do not protect developing winter bees from mites!!!!

"Factor in colony loss during overwintering?"

Cage longevity

Gravity feeders:
50:50 sugar solution & water

Newly emerged adults were counted, color-tagged

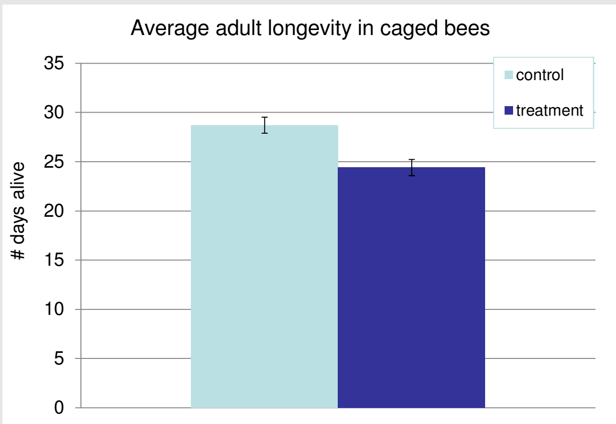
Cages were kept in incubators (30°C) without light

Mortality recorded daily

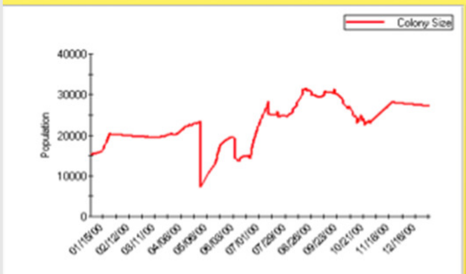
Testor's enamel

Cage longevity results

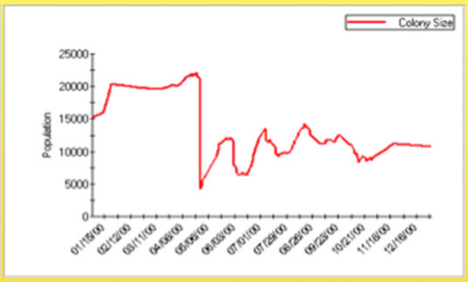
Adult control bees outlived adult treatment bees in the cage by 4 days (p<0.005)



What a Difference 4 Days Can Make



Workers live for 35 days
(21 days in the hive 14 days foraging)



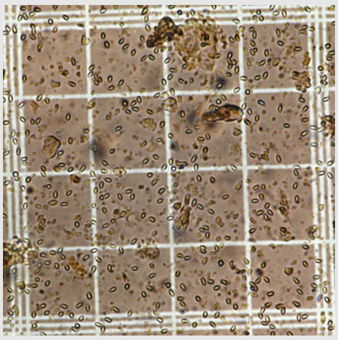
Workers live for 31 days
(17 days in the hive 14 days foraging)

Simulation results from the VARROAPOP Model

Study 3: Susceptibility experiments

Wu et al. (2012) J Invertebr Pathol. 109:326-329

Examine possible susceptibility N*osema* infection due to developmental exposure to pesticide residues



Collaboration with Matthew Smart

Colony 295:
formic acid acaricide,
no *Nosema* inoculant

(low *Varroa*, normal *Nosema*)

Colony 103:
no acaricide,
Nosema inoculant

(high *Varroa*, high *Nosema*)

-painted newly emerged worker bees (control & treatment) and put into colonies 295 & 103.

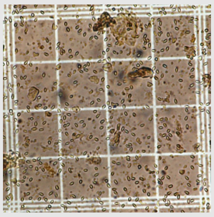
-sample 20 bees from each treatment and colony:
(week 2, 3, 4 post-release)

-control & treatment bees tested for *Nosema* levels.

-no difference in *Varroa* mite levels between treatments.

	Active ingredient	**Purpose**	**Chemical family**	**Toxicity to bees**	**Detection** (ng/g)	**LOD (ng/g)**
Comb Y	2,4 Dimethylphenyl formamide (DMPF)	Metabolite	Amidine		142	4
	Chlorpyrifos	INSECT	OP	High	8.5	1
	Coumaphos	INSECT	OP	Moderate	7230	1
	Coumaphos oxon	Metabolite	OP		231	5
	Endosulfan I	INSECT	OC	Mod	2.1	1
	Endosulfan II	INSECT	OC	Mod	1.6	1
	Esfenvalerate	INSECT	PYR	High	12.3	1
	Fluvalinate	INSECT	PYR	High	6800	1
	Phosalone	INSECT	OP	Mod	31.7	1
	THPI	Metabolite	Thiophthalimide		98.7	50
Comb G	2,4 Dimethylphenyl formamide (DMPF)	Metabolite	Amidine		147	4
	Coumaphos	INSECT	OP	Mod	281	1
	Coumaphos oxon	Metabolite	OP		10.2	5
	Chlorothalonil	FUNG	Chloronitrile		65.7	1
	Fluvalinate	INSECT	PYR	High	11280	1
	Permethrin total	INSECT	PYR	High	103	10
	Pyrethrins	INSECT	PYR	High	229	50

Characteristics of pesticide residues detected in treatment combs Y & G:
(OC, organochlorine; OP, organophosphate; PYR, pyrethroid; Mod, moderately toxic)



Effect of *Nosema* inoculants on spore level

Mean spore level	Colony 295	Colony 103 (added Nosema)
Control (G)	1,136	62,281
Treatment (comb G)	51,250	3,164,130
Control (Y)	3,488	170,212
Treatment (comb Y)	41,000	668,333

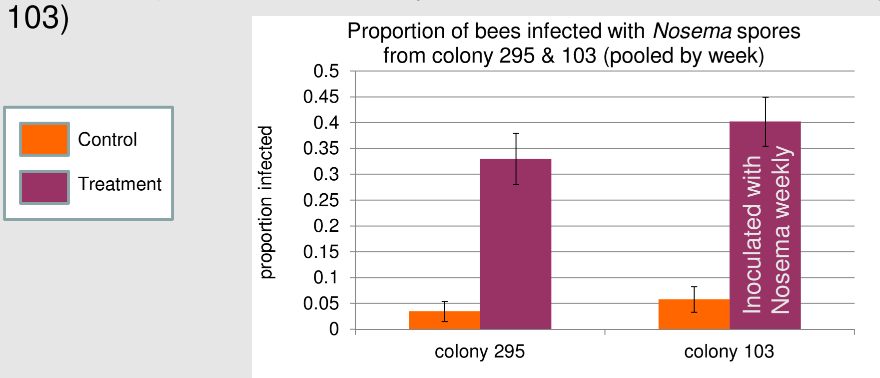
Colonies with Nosema inoculant added exhibited higher Nosema levels as expected

Nosema susceptibility

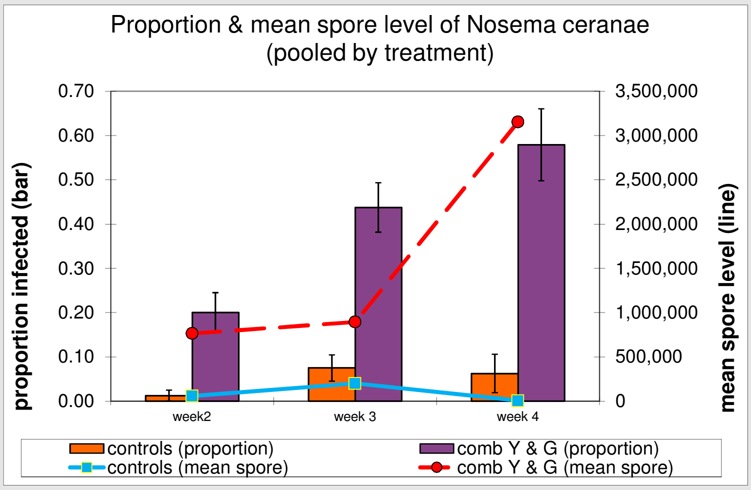
Bees individually analyzed for *Nosema ceranae* spores (n=473)

Overall proportion of infected treatment bees was higher in both colonies

There's no difference in proportion of infected bees between colonies! (even with 50 mil spores added each week to colony 103)



Nosema ceranae susceptibility



Proportion of infected was higher all weeks for "dirty" comb Y & G (infection occurred in younger bees!)

Proportion infected & mean spore count increased over time for bees reared in "dirty" combs Y & G

Summary of findings

Pesticide load survey - 75 different pesticides in brood comb (high of 22 in a single comb)

Brood effects – higher larval mortality, evidence for delayed development and adult emergence

Adult longevity – reduced by 4 days

Susceptibility tests - increased susceptibility to *Nosema;* higher proportion of infected bees & infection at younger age

Final thoughts

Pesticide load survey - synergistic interactions likely; chronic/long term effects unclear, effects from metabolites & fungicides unclear

Brood effects – less efficient brood production and care, over-worked queens, reproductive advantage for *Varroa* mites

Adult longevity –shortened lifespan increases colony need for brood production, pre-mature shifts in hive roles

Susceptibility tests - *Nosema* infection associated with immuno-suppressed bees? Stress related

Use Varroa treatments that do not accumulate in the hive

Hard treatments: synthetic or inorganic compounds

Soft treatments: natural or organic compounds (can still be toxic to bees)

Pesticide class	Active ingredient	Brand name
pyrethroid	Tau-fluvalinate	Apistan, Hivestan
organophosphate	Coumaphos	Checkmite+
formamidine	Amitraz	Apivar, Tactic
essential oil	Thymol	Apiguard
organic acid	Formic acid	Miteaway
organic acid	Hop beta acids	Hopguard
organic acid	Oxalic acid	
Grease patties with essential oils		
Food grade mineral oil mixtures		

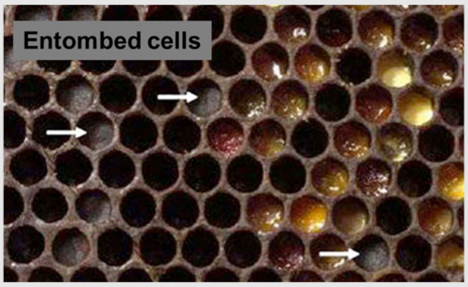
Comb replacement

Benefits
- "diluting effect"
- ↓ # nosema spores, AFB spores, etc.
- ↓ risk of mite resistance
- ↓ synergy (coumaphos and fluvalinate)
- Improve efficacy of future treatments

Disadvantages
- costly
- usually do not see immediate results
- need for replacement varies
- "diluting effect" will not be pesticide-free

Signs to look out for

- Old dark thick comb
- Mark frames when brood diseases have been observed
- Multiple entombed pollen cells
- Scratch & sniff tests



Current research at UNL

- Integrated Pesticide Management for beekeepers
- Impacts of pesticide residues on Varroa fecundity
- Monitoring for abnormal bee losses (dead bee traps)
- Reporting bee incidents (improvements to reporting)
- Comb replacement recommendations
- Deactivation of pesticide residues in comb

UNIVERSITY OF Nebraska Lincoln

Monitor for pesticide incidents

Dead bee traps

Easy to construct
Use to monitor abnormal loss of bees
Diagnose bees in traps

Reporting Bee Incidents

- State Agencies called to investigate
- High mortality in short period
- Samples in-hive & plants, soil, water
- Rule out other pests & pathogens

Problems:

- Mistrust/poor history
- Dwindling population (spiral effect)
- Unaware for weeks after initial event
- Too late for reliable residue sampling
- Weakened colonies have pests & pathogens

Reporting Bee Incidents

Need:

- Work with regulatory agencies
- To redefine examination criteria
  - Lethal → sub-lethal measures
  - Direct effects → indirect effects
- Improve metrics that can inform beekeepers of problem at start (rather then end of colony decline)
- Improve record keeping for colonies
- Public pesticide usage records/map
  - -Used to estimate exposure & risk
  - -Can help identify potential toxins for pesticide testing

This study illustrates that pesticides can have *subtle, sub-lethal,* & *indirect* effects that can have serious colony level consequences.

QUESTIONS ???

Take home message:

Regular comb replacement

Monitor mite levels
Use softer organic acid
Appropriately timed treatments

Monitor for bee losses

Record information:
(date, colony, health status, mite level, treatments)

Acknowledgments

Washington State University (2007-2010)

Drs. Steve Sheppard, Carol Anelli, John Stark

Tim Lawerence, Devrim Oskay, Beth Kahkonen, Kirsten Northfield

Matthew Smart, Sam Hapke, Ben Horwath, Debbie Delaney, Brandon Hopkins, Natalie Boyle

Edward Meyers, Charlie Cosler, Avery Nelson, Jen Vieyra, Terry Miller, Laura Leitz

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