Notes from the Lab:
The Latest Bee Science Distilled

by Scott McArt

A new high-throughput method to assess risk from pesticide co-exposures

Honey bees are almost always exposed to pesticides. For example, in a recent survey of 198 beekeeper hives throughout New York, we found pesticides in comb wax from 197 of the hives (Wheeler et al. 2018). On average, wax from the hives contained 6 different pesticides. And this is actually low pesticide exposure compared to some contexts. For example, when we screened bee bread from colonies conducting New York apple pollination in 2019, we found an average of 14 pesticides, with bee bread from some hives containing 23 different pesticides (Zhao et al., *in prep*).

The fact that pesticide co-exposures are so common poses a major problem for risk assessment by regulatory agencies such as the U.S. Environmental Protection Agency. Those agencies must determine whether risk from particular pesticides is high enough to warrant restrictions on their use. But currently, the EPA and other regulatory agencies only consider risk from individual pesticides, not combinations. This gives an incomplete picture of risk since 1) co-exposures to bees are ubiquitous, and 2) we know that some pesticides interact with each other (i.e., synergize) such that the blend is much more toxic than the individual pesticides on their own.

But how can we possibly get a handle on risk from co-exposures when honey bees potentially encounter hundreds of different pesticides? Some quick math shows that if there are 100 different pesticides that a honey bee might encounter, we'd need to screen 4,950 pesticide combinations to understand risk from dual exposures. If we want to assess risk from combinations of 3-pesticide exposures, that's 161,700 assays. And what about risk from combinations of 14 different pesticides, which is the average number of pesticides we found in bee bread from hives doing New York apple pollination? That's 44,186,942,677,323,600 assays. Good luck!

Clearly we need a quick and efficient way to screen honey bees for risk from pesticide combinations if we're going to start to get a handle on this important issue. This is the topic for our thirty-ninth Notes from the Lab, where we summarize **"Pesticide risk assessment at the molecular level using honey bee cytochrome P450 enzymes: A complementary approach,"** written by Julian Haas and Ralf Nauen and published in *Environment International* [2021, 147:106372].
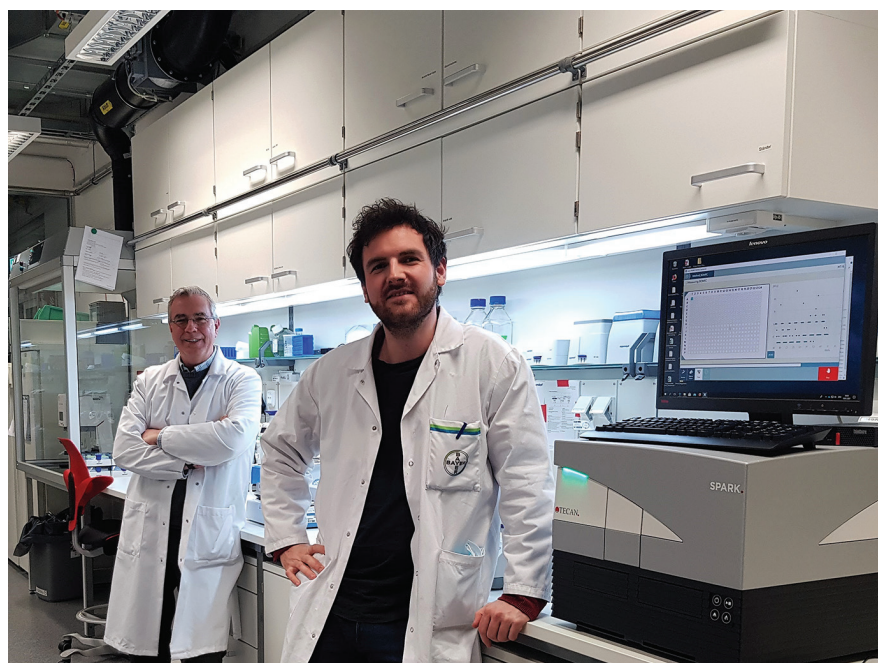

Photo 1: *Authors Julian Haas and Ralf Nauen in the lab and getting ready to do a lot of pipetting*

For their study, Haas and Nauen (Photo 1) took an approach that's similar to how drugs for humans are initially screened in the lab to determine whether drug-drug interactions have the potential to occur. Specifically, they assessed how well honey bee detoxification enzymes performed at breaking down pesticides into nontoxic products using 384-well microplates (Photo 2). Once they knew their assays were working well, they then tested the same pesticides on live honey bees and compared their high-throughput microplate assay results to what happened with the bees.

The basic concept of the microplate assay is shown in Figure 1. There are a lot of acronyms and symbols in the figure, but bear with me — it's actually pretty easy to explain and
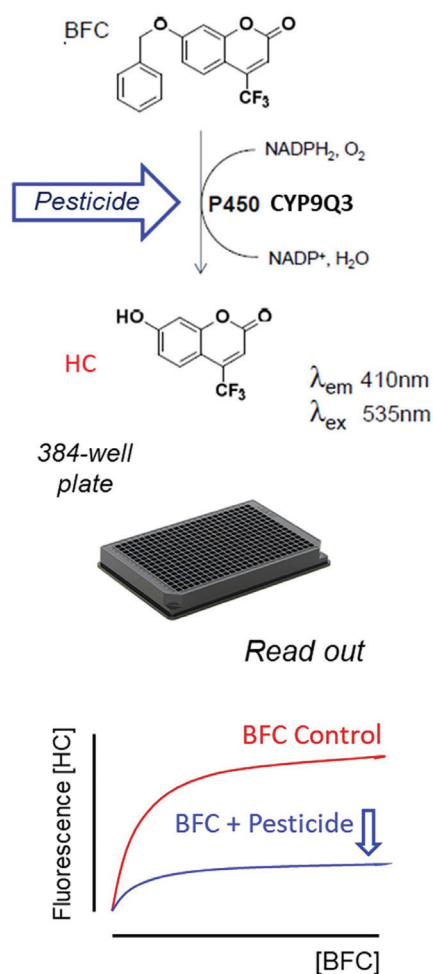
Fluorescence based assay

Figure 1: *Basic principle of the fluorescence-based screening assay measuring the pesticide-mediated inhibition of 7-hydroxy-4-(trifluoromethyl)coumarin (HC) formation by the honey bee CYP9Q3 detoxification enzyme*

provides a nice overview of how the authors' microplate assay works.

Starting at the top, the important point is that BFC (the molecule at the top) can potentially be broken down to HC (the molecule below the arrow) in the presence of the detoxification enzyme P450 CYP9Q3. However, this enzyme isn't able to convert as much BFC to HC in the presence of some pesticides (shown via the blue arrow). This could be for either of two reasons. First, the pesticide may compete with BFC for access to the detoxification enzyme (because it is also detoxified by that enzyme!). Such is the case for the neonicotinoid insecticides thiacloprid and acetamiprid, which are detoxified by CYP9Q3 in honey bees. Alternatively, a pesticide may inhibit the functioning of the enzyme, making it less efficient at detoxifying other things. As we will see later, such is the case for some fungicides, which inhibit CYP9Q3 and therefore interfere with the ability of honey bees to detoxify thiacloprid and acetamiprid.

Now move to the figure below the picture of the 384-well plate. This is what the authors monitored in the microplate assays: the amount of HC produced (measured via fluorescence) as more BFC was added in the presence of the CYP9Q3 detoxification enzyme. The blue curve is below the red curve, meaning less HC was produced in the presence of the pesticide. This indicates the pesticide either competes with or inhibits the CYP9Q3 detoxification enzyme.

OK, now that we're all on the same page with the authors' slick microplate assay, let's get to what they tested. First, Haas and Nauen assessed whether honey bee detoxification enzyme CYP9Q3 showed evidence of

detoxifying multiple neonicotinoid insecticides (thiacloprid, acetamiprid, imidacloprid, thiamethoxam) and a pyrethroid insecticide (*tau*-fluvalinate, which is also used as varroacide). Next, they tested whether several different fungicides (triflumizole, propiconazole, triadimefon, epoxiconazole, uniconazole, prothioconazole, prochloraz, and azoxystrobin) interfered with the detoxification enzyme. Finally, the authors performed LD50 studies with two of the neonicotinoids (thiacloprid and acetamiprid) on their own or via co-exposure with the fungicides. These LD50 bioassay data were then directly compared to the microplate enzyme activity results.

So, what did they find? Did the microplate assay show that insecticides are broken down by the CYP9Q3 detoxification enzyme? Yes and no. Haas and Nauen found evidence that thiacloprid, acetamiprid and *tau*-fluvalinate were broken down by CYP9Q3, but imidacloprid and thiamethoxam were not broken down by this enzyme.

What about the fungicides? Did they inhibit the CYP9Q3 detoxification enzyme? Yes and no. And this where Haas and Nauen's study really starts to break ground. As seen in Figure 2C, two of the fungicides (propiconazole and prochloraz, shown in orange and red) strongly inhibited the CYP9Q3 detoxification enzyme compared to controls (shown in black). But one of the fungicides (prothioconazole, shown in green) did not inhibit CYP9Q3. In other words, all fungicides are not created equal when it comes to inhibiting detoxification enzymes.

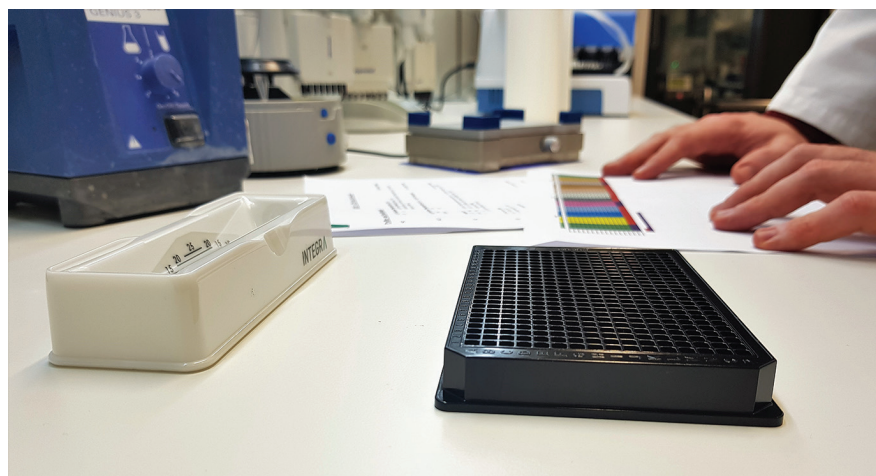**What about the LD50 results with bees? Was there evidence of syner-**

Photo 2: *One of the 384-well microplates that was used to screen pesticide combinations for their toxicity to honey bees*

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gism between fungicides and insecticides? Yes. As can be seen in Figure 2A and B, the similarity to the microplate results is striking. When honey bees were co-exposed to thiacloprid and propiconazole or prochloraz (orange and red lines in Figure 2A), the blend was ~700 times more toxic than when thiacloprid exposure occurred on its own (black line). Co-exposure to acetamiprid and propiconazole or prochloraz (orange and red lines in Figure 2B) led to the blend being ~200 times more toxic than when acetamiprid exposure occurred on its own (black line). Similar to the microplate results, co-exposure to prothioconazole (green lines) did not increase the toxicity of thiacloprid or acetamiprid.

Again, the LD50 bioassays show that not all fungicides are created equal. Co-exposure with propiconazole or prochloraz substantially increased the toxicity of thiacloprid and acetamiprid, while co-exposure with prothioconazole did not.

Well this is really interesting and a bit scary. Does the microplate assay predict all fungicide-insecticide synergisms? As seen in Figure 2D, the microplate assay with CYP9Q3 is strongly predictive of synergisms between acetamiprid and eight fungicides that were assessed via LD50 assays with honey bees. The authors also tested one more detoxification enzyme — CYP9Q2 — and it was found to have an even better correlation with the honey bee mortality data.

Haas and Nauen's correlation is very exciting for people who want to improve our knowledge regarding pesticide risk to bees. Because their microplate assay has high-throughput capability, it has the potential to rapidly screen many pesticide combinations. Given the extremely strong synergisms between some pesticides in this study (exposure to prochloraz increased the acute toxicity of thiacloprid to honey bees by ~700-fold!), along with knowledge that co-exposures to honey bees are ubiquitous, it's perhaps an understatement to say this type of microplate screening data would benefit regulatory agencies such as the EPA.

That said, expanding the assay to include additional detoxification enzymes and other modes of action is likely to be important. The CYP9Q3 detoxification enzyme used in this study is clearly important for the neonicotinoid insecticides thiacloprid and acetamiprid, but it had little im-
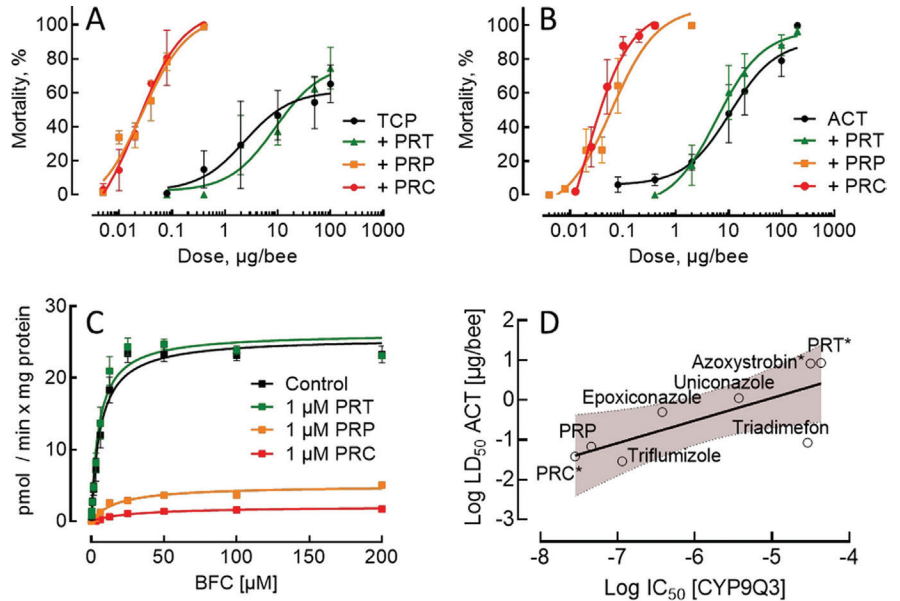

Figure 2: Dose-response relationship and synergism of (A) thiacloprid (TCP) and (B) acetamiprid (ACT) toxicity when topically applied to honey bees either alone or pre-treated with the azole fungicides prothioconazole (PRT), propiconazole (PRP) and prochloraz (PRC). Data are mean values ± SEM (n = 2-4). (C) Fluorescence-based CYP9Q3 inhibition assay with 1 µM of different azole fungicides. (D) Pearson correlation analysis (r = 0.76) between in vitro IC50 values and in vivo LD50 values obtained from CYP9Q3 fungicide inhibition assays and honey bee acute contact bioassays with acetamiprid in combination with fungicides, respectively.

pact on imidacloprid and thiamethoxam. And what about other insecticide classes, such as organophosphates and carbamates, or recently launched chemical classes that possess lower acute honey bee toxicity? Are there other enzyme or non-enzyme assays that work for screening co-exposures with those pesticides?

Haas and Nauen have opened the door to a very important topic; the next step is to see just how much is possible so we can improve risk assessments for our bees.

Until next time, bee well and do good work.

Scott McArt

Reference:
Haas, J. and R. Nauen. 2021. Pesticide risk assessment at the molecular level using honey bee cytochrome P450 enzymes: A complementary approach. *Environment International* 147:106372. **https://doi.org/10.1016/j.envint.2020.106372**

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