Interaction Between NaCl and Insecticide

* Continuous dependent variable of total growth (AUC).
* Categorical independent variable (treatment). Treatments are levels in a factor.
* Two insecticides, two concentrations for each (5µM and 5mM), with and without salt. Also, no salt (control) and salt.
* Salt is 0.5M NaCl.
* Ten levels in factor
  1. salt
  2. no salt
  3. thiacloprid 5µM (low)
  4. thiacloprid 5mM (high)
  5. acetamiprid 5µM (low)
  6. acetamiprid 5mM (high)
  7. thiacloprid 5µM + salt (low)
  8. thiacloprid 5mM + salt (high)
  9. acetamiprid 5µM + salt (low)
  10. acetamiprid 5mM + salt (high)
* With 9 replicates each the whole expt can fit all on one plate.
* Do three blanks with media and three blanks with NaCl media.

Are the effects of the two stressors additive or synergistic? If the effects are synergistic this improves the viability of the metabolism screen. If synergistic, by combining salt with insecticide the effect of insecticide alone has been amplified. This amplification provides a larger effect to be reversed if insecticide metabolism by a recombinant pollinator enzyme occurs.

Biological Interpretation

The main question from my results is why don’t most of the insecticides have an effect? Is it due to a lack of intracellular targets, or because their effective intracellular concentration is low, resulting from the action of efflux pumps in the plasma membrane? Can the proposed experiment lend support to either scenario? Can the effects of insecticide alone be explained by studying their effect in combination with NaCl?

Outcomes

Salt alone + insecticide alone = salt + insecticide together (additive)

Salt alone + insecticide alone < salt + insecticide together (synergistic)

Yannick’s argument – For insecticides that had no effect in isolation (e.g. acetamiprid)

*If additive result, then this shows that the insecticide (alone) enters the cell but has no effect.*

*If synergistic result, then small, previously undetected insecticide (alone) effect has been revealed through its interaction with another stressor. Shows that the small effect of the insecticide in isolation is due to efflux not a lack of intracellular targets.*

*Therefore, this experiment is an indirect way of looking at intracellular effect of the insecticide in isolation.*

This wasn’t his argument. I misunderstood. It was simply that if there is a synergistic effect between NaCl and insecticide then insecticide is having some form of effect (albeit in tandem with another stressor).

My argument –

If additive result, this only shows that there is no interaction between NaCl and insecticide stress. Nothing regarding the insecticide only intracellular effect can be concluded from this result.

If synergistic result, this only shows there is an interaction between the two stressors. Again, nothing can be inferred about the intracellular status of yeast for the insecticide only treatment.

My reasoning – There is an alternative explanation to yannick’s. For the synergistic result, this wouldn’t necessarily be uncovering a previously undetected, but present/potential, insecticide only effect. The insecticide in isolation may be present at high intracellular concentrations and be benign, and only have an effect when combined with NaCl. There are multiple examples of where two substances in isolation are harmless, but when combined are toxic, in the literature1. It’s possible that treatment with the insecticide in isolation leads to a low intracellular concentration (due to poor influx or rapid efflux) and a small effect, and combination with NaCl either facilitates insecticide entry or heightens the existing effects driven by the low intracellular insecticide concentration. But this isn’t the only explanation. Furthermore, the additive result would also be inconclusive: maybe, on treatment with only the insecticide, intracellular insecticide concentration isn’t reflective of the media concentration due to poor influx, and NaCl treatment doesn’t remedy this. In this case, the insecticide in combination with NaCl would still have no effect but may if it was present intracellularly. An alternative explanation is the insecticide in isolation treatment results in a low intracellular concentration (due to poor influx or rapid efflux) and a small, undetectable effect, but there is no interaction between this and the effect of NaCl.

An alternative/additional experiment would be to try and measure intracellular insecticide concentration using quantitative mass spec. This would at least clarify if the lack of effect for most insecticides is due to a low intracellular concentration. Of course, if their intracellular concentration is low this wouldn’t answer if they would have an effect if it were high. Moreover, my results would more relevant if there was only a low intracellular concentration. For example, with Thiacloprid I could then argue that the concentrations tested were relevant as the intracellular concentration was actually similar to those encountered in agricultural settings (for soil microbes at least). Of course, a counter argument exists where the concentrations encountered in field realistic settings are actually present at much lower levels intracellularly too.

Response to gene expression study

Yannick mentioned performing it anyway with yeast+NaCl as a control and yeast+NaCl+insecticide as the treatment. This wouldn’t necessarily inform on insecticide only effects. The observed effects may only be present in the combination of the two stressors. The interaction between insecticide and salt is only interesting for marine fungi. This isn’t what I’m interested in.

**Above was a criticism of something Yannick didn’t mean. Useful if it comes up again but otherwise ignore.**

Other Options for Investigating Off Target Effects

Choose an insect cell line (potentially Sf9 (Sf21 substrain) derived from *Spodoptera frugiperda*, fall army worm moth*)* or High Five (or another cell line derived from *Trichoplusia ni*, cabbage looper) that doesn’t express nAchR (check existing RNA-seq data to see if nAchR expressed). Closer taxonomically to species of interest and (probably) won’t possess detoxification battery of plasma membrane transporters. Yeast are single cell organisms and require efficient detoxication mechanisms. Insect cell lines, on the other hand, unless derived from tissues associated with detoxification, wouldn’t be expected to. For Sf9 there is a whole genome sequence available and suspension culture is possible3,4. Genome assembly for *Trichoplusia ni* Tni-FNL insect cell line available and was originally selected for suspension growth5. Suspension important for growth measurements using a spectrophotometer. Even this isn’t a very strong idea (see below).

Look at effects in *S.pombe*. Evolutionary distance between *S.pombe* and *S.cerevisiae* similar to that of either one to mammals (Hoffman 2015 Genetics). For *S.pombe*, 338 proteins conserved in vertebrates but absent in *S.cerevisiae*. In comparison, 179 genes conserved between *S.cerevisiae* and vertebrates that are absent from *S.pombe*. Do a similar analysis to above but for *B.terrestris* and *A.mellifera* instead of vertebrates.

Measure intracellular concentration to display concentration causing observed effect.

Explore the differences between formulations and active ingredients in yeast. If active formulations inhibit growth more than the active ingredients alone then there are some off-target effects driven by the additives. I could also perform a study like this in bees, which would be my preference if I’m honest. There is evidence that formulations are more toxic to bees than pure insecticides6–8. Investigating this at the transcriptomic level would uncover the additional effects, driven by “inert” additives/co-formulants, responsible for the toxicity gap.

Acute field realistic exposure compared to my concentrations

Alicja’s experiment is using an acute dose of 25ppb. I have found that Thiacloprid causes small but significant growth inhibition in yeast at 5mM. 5mM = 1263600 ppb. So, I am testing approximately x50000 the concentration of the acute, field realistic dose. Therefore, if intracellular concentration was 1% of the media concentration, it would still be x500 the acute, field realistic dose. This isn’t environmentally relevant. One study has already looked at the effects of insecticides on an insecticide cell line, Sf9. They reported a IC20 of 61.6µM for acetamiprid9. This is equal to 13710ppb. A 20% growth inhibition required a concentration x548.4 greater than the acute dose in Alicja’s experiment. Taken together, the results imply that the off-target effects of neonicotinoids are negligible at field realistic exposures and aren’t worthy of further investigation.

Response to Yannick Wanting to Use Yeast for Further Expts

Yeast was useful for separating off target effects from those mediated through the primary molecular target. For any further experiments this would no longer be the aim. Instead, if the effects of co-formulants was explored in more detail (formulations vs active ingredients), using yeast would be counterintuitive because not all the effects in the species of interest would be detected. To study the effects of formulations and how they differ from active ingredients in isolation, the aim is to characterise the difference in overall effects between the two, including those mediated through nAchR (or other primary molecular target). Therefore, testing on yeast would yield an incomplete picture. In fact, the very reason yeast was selected in the first place (lack of nAchR) is what makes it unsuitable for any subsequent studies that are interested in effects as a whole, not just off target effects. My previous experiment suggests there are essentially no insecticide effects in yeast, making this model organism unsuitable for further insecticide testing.

Interested in the overall effect on active ingredient in isolation vs overall effect of formulation. The difference between the two is co-formulants + the interaction between co-formulants and insecticide. What are the gene expression changes caused by co-formulants + the interaction between co-formulants and insecticide? Link to fitness effects as well. TYPE UP REST OF HAND WRITTEN NOTES.

Related Points

The power of the metabolism screen lies in being able to screen many insecticides across one enzyme. Not many enzymes across one insecticide. This would cost more, take longer (cloning) and less interesting questions could be answered. Also, if yeast is sensitive to many insecticides, we know that we can expand the number of enzymes in the screen. Insecticides are the big if. For synergistic interactions between insecticides and salt to be useful they have to present for many insecticides and be strong enough to produce a large, reversible effect.

In relation to off target effects, if no more experiments are undertaken, the negative result is more conclusive. From the current experiment can strongly suggest that there are no off target effects relating to biological processes conserved between yeast and metazoans. There may still be off target effects in pollinators, but these don’t involve processes conserved between yeast and metazoans. If there had been effects there was nothing to say the MoA involved conserved processes, instead it could have been a yeast specific toxicity. Also, the thiacloprid effect suggests intracellular activity. How could thiacloprid exert an extracellular effect? Potentially osmotic stress, but if this were true then surely this would have been observed for other insecticides at the same concentration. If thiacloprid intracellular, likely other insecticides with similar structures also entered the cell. Therefore, their intracellulars concentrations had no effect and didn’t perturb fundamental, ancient eukaryotic pathways.

How does insecticide discovery work? Screen compounds against target. How do they test for off target effects?

Next Steps

Look at yeast sensitivity to insecticides in another way through a Halo assay. Large volumes of high insecticide concentrations required is prohibitive for a spot test. Becomes so expensive you may as well conduct mass spec.

Repeat sensitivity test with PDR1/PDR3 knockout. Is there a larger effect to reverse regards the metabolism screen? At what concentration range are effects now seen? Much lower and therefore potentially relevant to *in vivo* exposure?

A worry for the metabolism screen is the reaction rate of the enzyme won’t be fast enough to change intracellular insecticide concentrations in comparison to the wildtype. Is there a way to calculate this? Would need to know insecticide influx rate. If much larger than enzyme reaction rate, then the enzyme will have little effect on intracellular insecticide concentrations. If, however, it is comparable to influx it will lower intracellular insecticide concentrations. Another issue is the enzyme product could be as toxic to yeast as the substrate!

If the metabolism screen does work then some really interesting questions can be answered. Could synthesise transgenic yeast each containing an ortholog of a detoxification enzyme. Could then compare the substrate profiles of the orthologs and see if there is a correlation between substrate profile similarity and evolutionary distance. Also, could assess how transferable results in one species are to close relatives. Could even lead to a prediction model if there was enough data (semi-quantitative too).

Flytox is an issue but this would have higher throughput if successful.

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