Differential Gene Expression of Yeast to Pesticides

* Which pesticides?
* Mixtures?
* What concentration? What are the soil concentrations of pesticide residues?
* Exposure time? Chronic or acute?
* How long will it take?
* RNA extraction process?

Useful for tracking molecular effects not mediated through primary target sites. Could be a simple model system for toxicity regulation testing. An easy first testing step for new pesticides to characterise their effects on eukaryotic cells and pathways that are conserved across many eukaryotic species.

Are regulators of chemical stress response activated?

Insights in secondary toxicity mechanisms.

Comparisons with existing DEG studies in bees could be made. Are their similar gene expression patterns on a particular subset of orthologous genes?

Which Pesticides?

Choose only pesticides with primary targets not present in yeast. No nAchR, acetylcholinesterase, ryanodine receptor (RyR) or Nav channels ([link](https://www.pnas.org/content/108/22/9154)). Yeast do have a TRP homolog, called YVC1, which is a voltage dependent cation selective channel (Na+, K+, Ca2+) ([link](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC35422/)). This has GO term of [sodium channel activity](https://www.yeastgenome.org/go/GO:0005272) based on [this](https://www.pnas.org/content/87/20/7824) (unsure if this could be a target of pyrethroids, read [more](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3386883/) [more](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6259924/)). However, *S.cerevisiae* does have a α1 (pore forming and voltage sensing) subunit Cav homolog ([CCH1](https://www.yeastgenome.org/locus/S000003449), [ref](https://mcb.asm.org/content/17/11/6339)). Nav probably descended from Cav ([link](https://www.pnas.org/content/108/22/9154)), their alpha subunits share a 4 domain structure with 6TM segements per domain. Consequently, pyrethroid activity has been reported against Cav channels ([link](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3218237/)). Pyrethroids interact with the alpha subunit of Nav ([link](https://europepmc.org/article/pmc/pmc1277854)). The alpha 1 subunit forms the pore and voltage sensing component of Cav and Nav. Other subunits act as regulators ([link](https://www.ebi.ac.uk/interpro/entry/InterPro/IPR005445/)).

There are homotetrameric 1x6TM bacterial Na channels, which consist of 4 repeat subunits, that are comparable to the 4x6TM eukaryotic Na channels (all one subunit, the alpha subunit). In bacterial sodium channel the pore selectivity filter has EEEE configuration (1 each from each subunit). This is the same/comparable to Cav channels (usually E/E/E/E or E/E/D/D) (1 residue from each domain). Selectivity for sodium, on the other hand, is based on the residues D/E/K/A in the pore. The closest homolog to the prokaryotic 1 × 6 TM Na-selective channels are the eukaryotic transient receptor potential (TRP) family of channels (yeast has TRP homolog in the form of YVC1). Members are usually non-selective or sodium selective channels, with exceptions, such as Catsper1 with an electronegative high field strength (HFS) sites which are key for conferring a calcium-selectivity (EEEE or EEDD).

Conclusion from this is pyrethroids will likely have secondary effects that have been partially explored in the current literature.

Neonicotinoids – cyanoimines (thiacloprid, acetamiprid), nitroimines (clothianidin, imidacloprid)

Organophosphates/chlorine - malathion, chlorpyrifos

Other nAchR modulators – sulfoxaflor, flupyradifurone

Pyrethroids (type 1) & (type 2) - cypermethrin (Type 2), tefluthrin (Type 1)

nAchR allosteric modulators – Spinosad

Diamides – chlorantraniliprole, cyantraniliprole

7 groups. 13 pesticides

Intra and inter group mixtures. Would be an appropriate second stage if the first stage is informative.

96h exposure to ecologically relevant concentrations of pesticides. Find info for ecologically relevant concentrations. This would test potential secondary effects activated at field realistic concentrations.

Concentration Options

Want to reveal transcriptomic effects at ecologically relevant concentrations. Each concentration will preferably be based on residue measurements of soil, nectar or pollen.

Two options: can have concentrations different for each insecticide to elucidate effects at residue levels found in vivo OR can have the same concentration for each insecticide, determined by a residue concentration ranges. Find concentration that is contained within the most insecticide residue recorded ranges. In the case where residue concentration for an insecticide doesn’t overlap with most frequent concentration for all insecticide residue ranges still apply this concentration.

This approach would be a better comparison of the absolute toxicity hazard between the insecticides. Not what the aim of this experiment is.

The first approach would be a better of profiling the secondary toxicity mechanisms / transcriptomic changes activated at field realistic concentrations.

Conclusion – First approach where the median concentration of recorded residue concentrations is applied. Will be different for each insecticide. Will elucidate relevant perturbations.

FIND RESIDUE CONCENTRATIONS FOR PESTICIDES AND RECORD MEDIANS.

From Colgan S1 table of grouped environmental concs of imidacloprid (<0.16-3.03ng/g or 0.2-39ppb) and clothianidin (<0.12-13.28ng/g or 0.5-13.9ppb). Find residue concentration values for all insecticides included and hope ranges overlap for all so 1 concentration can be picked.

Or could use an average (median) of maximal residue levels on EU website. Or half maximal residue levels. For example, honey is 0.05 mg/kg (or 50 ng/g).

Would have to work in g/ml and convert to (ng/g) / (ppb) at the end.

Mass of solute in 1L (g) / (Mass of solute in L + Mass of solvent) (g) = Mass of solute per gram of solution. Ppb = ng/g

What is a sensible g/ml? Colgan used 7.5 × 10−9 g/ml. In sucrose solution this worked out as 6.47 ppb. Obviously be different for YPD (be more as YPD will have a lower mass 7.23 ppb)

Example calculation:

YPD or YP-GAL (rich media)

To 350ml H2O add…

5g Bacto Yeast Extract

10g Bacto Peptone

Optional: 10g Bacto agar - for plates

Autoclave, then add...

50ml 20% Glucose OR 50ml 20% Galactose

100ml sterile H2O

For 1L 900 ml H20 + 10g Yeast + 20g Peptone + 100ml 20% m/v glucose

Mass of 100ml 20% m/v glucose:

d = m/v , d = 1.56 g/ml , m = 20g

v = m/d = 20g/1.56gml-1 = 12.82ml

If total volume 100ml then 100ml-12.82ml = 87.18ml H20 to make up to 100ml.

Therefore, for 100ml of 20% m/v glucose, mass = 87.18g + 20g = 107.18g

1L of YPD = 900g H20 + 10g Yeast + 20g Peptone + 107.18g 20% m/v glucose

= 1037.18g

For example, if 7.5x10-9g/ml concentration of Imidacloprid used:

Solute = 7.5x10-6g/l

Solution = Solute + Solvent = 7.5x10-6g/l + 1037.18g/l

For mass of insecticide solute in 1g of solution =

(7.5x10-6g/l)/ (7.5x10-6g/l + 1037.18g/l) = 7.23x10-9g solute /g solution OR 7.23ng solute/g solution OR 7.23 ppb.

In YPD a conc of 1x10-8g/ml would equate to 9.64ppb. If concentration the same for all the insecticides would be a fair way to compare their toxicity and magnitude of effect on the transcriptome.

Work in M (mol/l). Then for each concentration same number of particles of each insecticide will be present for each concentration. Fairer comparison if 1 particle is active unit. Effect exerted as a function of particles not as a function of mass. Therefore, compare same number of particles not same mass.

Lab time

Yeast Seeding and Insecticide Exposure

* Grow yeast overnight. Record when yeast cells were harvested (log growth phase with approximate OD600 reading of 0.25).
* Have to check solubility but at such low concentrations should be fine in aqueous solution.
* If 13 pesticides and 4 repeats then n = 56 including 4 controls.

RNA Extraction

Pellet harvested by centrifugation and washed with DEPC treated water ([link](https://www.sciencedirect.com/science/article/pii/S0168160517302982?via%3Dihub#s0010)). At this stage can freeze at -80.

Yeast cells need to be purified ([link](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC330876/pdf/nar00194-0236.pdf)). Hot phenol method or beads ([link](https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-019-5511-x#Sec14))?

Afterwards following Colgan protocol should be fine ([link](https://onlinelibrary.wiley.com/doi/10.1111/mec.15047)).

Day 1

* Make some YPD.
* Create insecticide stock solutions. Can they all be dissolved in acetone to 1.0x10-3 g/ml?
* Grow some yeast colonies overnight.

Day 2

* Record when yeast cells were harvested (log growth phase with approximate OD600 reading of 0.25).
* Generate insecticide working solutions (in media). Make it so that when media containing yeast is added final desired concentration is achieved.
* How much yeast for each sample to ensure enough RNA can be extracted for sequencing (1.5 µg)? RNeasy kit recommends 2x106 – 5x107. If no information about RNA content don’t start with anymore than 2x107 yeast cells. Aim for 1x107cells, which will yield roughly 25 μl (p17). Unless spectrophotometer brands listed in RNeasy guide ([p44](https://qmulprod-my.sharepoint.com/personal/btw905_qmul_ac_uk/_layouts/15/Doc.aspx?sourcedoc=%7b5961a1ba-e43a-496d-a174-be35e869a6b3%7d&action=view&wd=target%28Biology%20Department.one%7Cee212005-4af1-4996-81ba-da7bda456799%2FRneasy%20mini%20kit%7Cbe22e1a5-1aa9-44b6-97bb-1742226934e4%2F%29)) used by lab will have to calibrate machine. Use a count slide ([unit 1.2 protocols etc](http://www.aun.edu.eg/molecular_biology/PCR(1)/Current%20Protocols%20in%20Mol.%20Biol..pdf))
* Treat yeast for 96 hours. Put back on shaker at 30oC. 48 individual eppendorfs in rack?

Day 6

* Centrifuge to extract yeast from solution and wash with DEPC treated water. Then Tri reagent added.
* At this point can freeze at -80oC.
* Sample disruption using FastPrep-96 high throughput homogeniser.
* RNA isolation and extraction using chloroform. ‘single-step method’ ([link](https://www.nature.com/articles/nprot.2006.83)).
* Wash with ethanol.
* Further purify using RNeasy MiniPrep kit (Qiagen, UK).
* Remove residual DNA using RNase‐free DNase I (Qiagen, UK).
* Quantified total RNA using a Qubit RNA Broad‐Range (BR) Assay kit (Invitrogen, UK).

Day 7

* Sequence library prep using Illumina TruSeq stranded mRNA library preparation kit.

Method Post Treatment

* The pellet was harvested by centrifugation (4000 rpm/min, 5 min) at 4 °C and washed with DEPC-treated water. The biomass was stored at − 80 °C until further treatment.
* Follow either of these methods ([link](https://qmulprod-my.sharepoint.com/personal/btw905_qmul_ac_uk/_layouts/15/Doc.aspx?sourcedoc=%7b5961a1ba-e43a-496d-a174-be35e869a6b3%7d&action=view&wd=target%28Biology%20Department.one%7Cee212005-4af1-4996-81ba-da7bda456799%2FPROTOCOL%20RNA%20extraction%20for%20S.invicta%20tissues%7Cf039ac37-f1fc-4b3e-8e76-175e9911f7b3%2F%29))([link](https://qmulprod-my.sharepoint.com/personal/btw905_qmul_ac_uk/_layouts/15/Doc.aspx?sourcedoc=%7b5961a1ba-e43a-496d-a174-be35e869a6b3%7d&action=view&wd=target%28Biology%20Department.one%7Cee212005-4af1-4996-81ba-da7bda456799%2FPROTOCOL%20RNA%20extraction%20TRIZOL%20Insects%7C11a0c456-a7b6-497f-abe1-2ace7095152f%2F%29)). Almost the same.
* Progress onto RNeasy miniprep method ([link](https://qmulprod-my.sharepoint.com/personal/btw905_qmul_ac_uk/_layouts/15/Doc.aspx?sourcedoc=%7b5961a1ba-e43a-496d-a174-be35e869a6b3%7d&action=view&wd=target%28Biology%20Department.one%7Cee212005-4af1-4996-81ba-da7bda456799%2FRneasy%20mini%20kit%7Cbe22e1a5-1aa9-44b6-97bb-1742226934e4%2F%29)). Sounds like initial steps of this are analogous previous step. Skip homogenisation and just centrifuge in order to remove nuclease free water? Then resuspend pellet in Buffer RLT? Then ethanol etc etc?
* Residual DNA can either be removed after RNA purification by a DNase digestion or during RNA purification using on-column DNase digestion using DNase I solution.
* Federico said “I typically follow the Qiagen RNeasy protocol to isolate total RNA from animal tissues, adding adding β-mercaptoethanol (β-ME) to Buffer RLT before use and with on-column DNase digestion using DNase I solution”.
* Instead of above could follow RNeasy guide from page 44. Specifically for yeast with optional on-column DNase digestion ([link](https://qmulprod-my.sharepoint.com/personal/btw905_qmul_ac_uk/_layouts/15/Doc.aspx?sourcedoc=%7b5961a1ba-e43a-496d-a174-be35e869a6b3%7d&action=view&wd=target%28Biology%20Department.one%7Cee212005-4af1-4996-81ba-da7bda456799%2FRneasy%20mini%20kit%7Cbe22e1a5-1aa9-44b6-97bb-1742226934e4%2F%29)).
* Once you have isolated total RNA, use a small volume of each extract to evaluate RNA integrity in a Bioanalyzer ([link](https://www.agilent.com/cs/library/applications/5989-1165EN.pdf))([link](https://www.agilent.com/en/product/automated-electrophoresis/bioanalyzer-systems/bioanalyzer-rna-kits-reagents/bioanalyzer-rna-analysis-228256)).
* Remove ribosomal RNA (rRNA), poly-A selection, but there are also rRNA depletion methods ([link](https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-018-4585-1)). Again, RNeasy kit is designed to enrich for mRNA and other RNA species >200nt (p17).
* The last part is the library preparation using either an Illumina TruSeq stranded mRNA kit or a NEB kit. Time consuming.

High Acute vs Low Chronic Exposure?

High Acute more likely to exhibit change in gene expression but at the cost of relevance. Concentrations required only relevant to poisoning or polluting accidents. High acute exposure could be used to compare transcriptomic profiles of different insecticides and see if family members have similar effects. Test the relevance of current IRAC classification system.

Counter-argument is that a model organism that contained the relevant primary molecular targets would be more suitable for this. Would provide information on off site targets/responses but easy counter argument is these off site targets/response aren’t interacted with/induced at recorded residue levels found in soil, apiaries, pollen and nectar samples. Best that could be said is that this approach would outline potential secondary toxicity mechanisms, but these may not be relevant.

Low Chronic Exposure is an ecologically relevant test. Could reveal off site targets that are interacted with at field realistic concentrations. Con is there may not be any measurable effects at such low concentrations.

My assessment is the aim of this project is to reveal off target effects of insecticides. Off target effects are dependent on the insecticide concentration. Therefore, a high acute dose isn’t representative of pollinator or other wildlife exposure in the wild via contamination. What is representative is low chronic exposure. (For example, hydrogen peroxide at low concentrations is a signalling molecule, at high concentrations a ROS).

If, however, I wanted to test overall secondary mechanisms of toxicity (how insecticides exert their effects on target species) I could use an acute, high dose. Also, an acute high dose approach could explore other applications of insecticides. But that isn’t the goal of this expt.

What did other studies do?

* We therefore investigated the immediate response of wild type BY4741 cells treated with CTBT. We used a dose 1/3rd of the minimal inhibitory concentration (MIC) (6 μg/ml) 2 μg/ml in a time curse of 5, 10, 20 and 40 min in liquid medium. Expression data were collected from duplicate arrays ([link](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2841119/#B29)). ([MIC](https://en.wikipedia.org/wiki/Minimum_inhibitory_concentration)).
* In addition to media components as described in Section 2.2, the test plates contained, 15 mM acetamiprid, 10 mM thiamethoxam, or 0.8% (v/v) DMSO and 0.9% (v/v) NMP. Concentrations of Mospilan, Actara and Confidor in screening experiments were equivalent to 5 mM acetamiprid, 20 mM imidacloprid and 20 mM thiamethoxam. The concentrations were selected based on the pre-determined semi-inhibitory (i.e. 20% growth inhibition in the wild-type strain) concentration and solubility ([link](https://www.sciencedirect.com/science/article/pii/S0045653513014975?via%3Dihub#s0010)).
* For transcriptional analysis, exponential growing cells were given exposure to an optimised concentration (1.3mM) lindane dissolved in DMSO for 2h and control cells were treated with DMSO alone ([link](https://www.jstage.jst.go.jp/article/cbij/3/1/3_1_12/_pdf/-char/ja)).
* The range of quinine concentrations tested was 1.5–1.7 g/L. Growth was inspected after incubation at 30°C for 2–4 days, depending on the severity of growth inhibition ([link](https://link.springer.com/article/10.1007%2Fs00438-011-0649-5)).
* A volume of 50 μl of 2 mg/ml SM in DMSO was then added onto the filter disc (31). The plates were incubated at 30°C for 24 h, and the diameter of the zone of growth inhibition was measured ([link](https://journals.physiology.org/doi/full/10.1152/physiolgenomics.2000.3.2.83?url_ver=Z39.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub%20%200pubmed)).
* Sample B, corresponding to sample A cell population 1.5 h following inoculation into fresh medium supplemented with 0.3 mM of 2,4-D, when the period of 2,4-D-induced death is approaching its end ([link](https://onlinelibrary.wiley.com/doi/epdf/10.1002/pmic.200401085)) ([link](https://academic.oup.com/femsyr/article/6/2/230/650542)).
* The sensitivity of 4700 homozygous deletion yeast strains was tested in the presence of sublethal doses of MPP+ (250μM) and paraquat (2000μM). These doses were derived from a serial dose response curve to define conditions in which wild-type yeast cells manifest 10% reduction in growth rate ([link](https://academic.oup.com/toxsci/article/95/1/182/1690987#87988027)) (fitness study, cool).

Next we analyzed the connection between transcript and phenotype profiles (Figure ​(Figure3A).3A). Generally, phenotypic display data do not have large overlaps with transcript profile data. This is due to the fact that pathways activate many target genes in parallel with sometime redundant functions. However, in some cases the overlap is informative since it points to exceptionally important nodes of stress resistance.

Transcriptomic analysis followed by chemogenic analysis on DEGs from transcriptomic analysis. Use transcriptomic analysis as an initial screen. Overlap will identify “exceptionally important nodes of secondary mechanisms”.

ICPMS to quantify intracellular insecticide concentration. If the amount can be calculated so can concentration. Can detect metal ions. Not so well established for insecticides. ICPMS can’t detect O and N as it’s not a closed system. Air intake makes analysing these elements impossible.

For each treatment regime do an extra 3 samples for mass spec analysis. Calculate cells per ml. Then wash cells, centrifuge, lyse cells to release intracellular pesticide, collect supernatant and analyse by UPLC-MS analysis. Metabolite extraction is performed via boiling ethanol or chloroform-methanol. Ask about appropriate MS technique. ([link](https://pubs.acs.org/doi/pdf/10.1021/ac900999t) for metabolite extraction from yeast) ([link](https://pubmed.ncbi.nlm.nih.gov/29576476/) for UPLC-MS use, manjon). Also, are MS spectra available for insecticides, not their metabolites (more likely).

Require stranded library - Third, the yeast transcriptional landscape is composed of bidirectional and overlapping transcripts that are embedded in a dense genome—this presents a challenge for RNA-seq and requires stranded libraries to capture complex genome architectures. ([link](https://www.nature.com/articles/s41564-018-0346-9))

Knowing field realistic concentrations is one thing, knowing intracellular/intratissue concentrations is another entirely. What is the final concentration of insecticides in insect cells/tissues is unknown. Same issue for yeast.

Questions for Peter?

Why harvest in log phase? So transcriptomes between samples are comparable. Controlling for inter-sample variability? Why not stationary phase? Quiescent yeast cells (typically studied in stationary-phase [SP] cultures) exhibit very low metabolic activity, including low rates of protein synthesis (Fuge et al., 1994 blue right-pointing triangle) and transcription (Choder, 1991 blue right-pointing triangle; Jona et al., 2000 blue right-pointing triangle). RNAses are abundant in these cells and have been found to copurify with poly(A)+ RNA (Gray et al., 2004 blue right-pointing triangle). The thick cell walls of stationary-phase cells impede cell lysis, making isolation of proteins and native protein complexes difficult. Nevertheless, the importance of the quiescent state in all organisms makes this a compelling area of research. ([link](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC532011/))

How to keep in log phase? Keep diluting yeast whilst maintaining concentration. This will keep returning it to log phase. Will keep the cycle going. (Lag -> expo -> stationary repeat when yeast diluted)

For high acute dose how long after dose to get a log phase sample? When in 0-4 hour window?