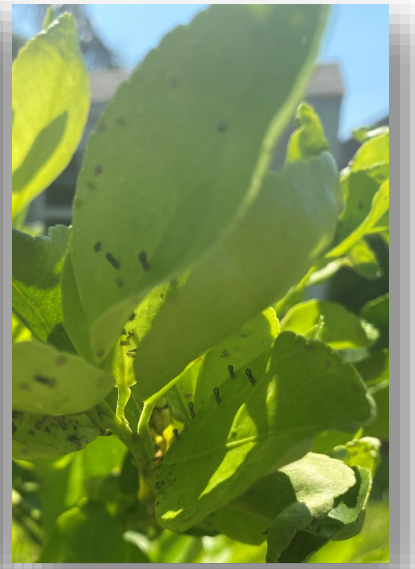
CROSS-BORDER THREAT SCREENING AND SUPPLY CHAIN DEFENSE

DEPARTMENT OF HOMELAND SECURITY
SCIENCE AND TECHNOLOGY CENTER OF EXCELLENCE

SUMMER RESEARCH INSTITUTE

AUGUST 4, 2023

CBTS Summer Research Institute
Final Student Fellow Presentations



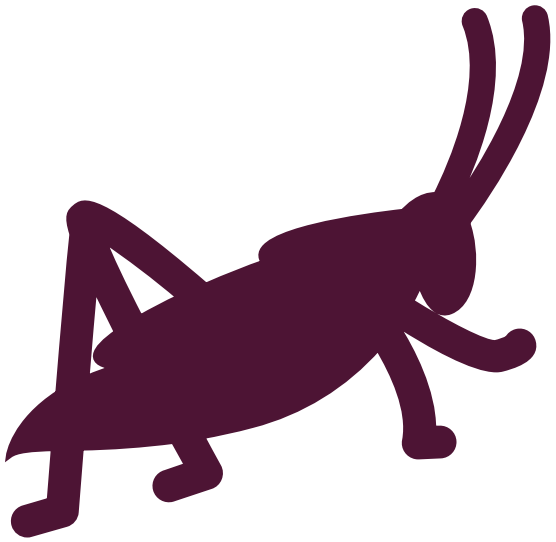
Presentations:

- Joseph Wilson – Hemolymph “blood” test: Raman detection of density-dependent differences in locusts
- Maximiliano Hernandez – Citrus Greening disease identification and development towards resistance against CLas infection
- Axell Rodriguez – Utilization of Atomic Force Microscopy-Infrared Spectroscopy to access secondary structure and stability of proteins
- Jocelyn Gutierrez – Temporal infection analysis of Tobacco Mosaic Virus (TMV) in *Nicotiana benthamiana*
- Aidan Holman – Identification of adult Ixodid ticks by RAMAN spectroscopy of their feces

HEMOLYMPH "BLOOD" TEST: RAMAN DETECTION OF DENSITY-DEPENDENT DIFFERENCES IN LOCUSTS

JOSEPH WILSON

OVERVIEW



Raman spectroscopy was tested as a novel method for the identification of population conditions in locusts, a type of grasshopper known for its ability to form swarms.

- Raman is a non-destructive and non-invasive way to perform chemical analysis on a sample
- If found to be accurate implementation could improve the ability to study phase polyphenism the phenomenon responsible for swarm formation in locusts
- The ability to prevent plagues of locusts and agricultural destruction in various areas around the world they cause relies on a deeper understanding of this phenomenon

BACKGROUND

LOCUST PHASE POLYPHENISM: PHENOTYPIC PLASTICITY

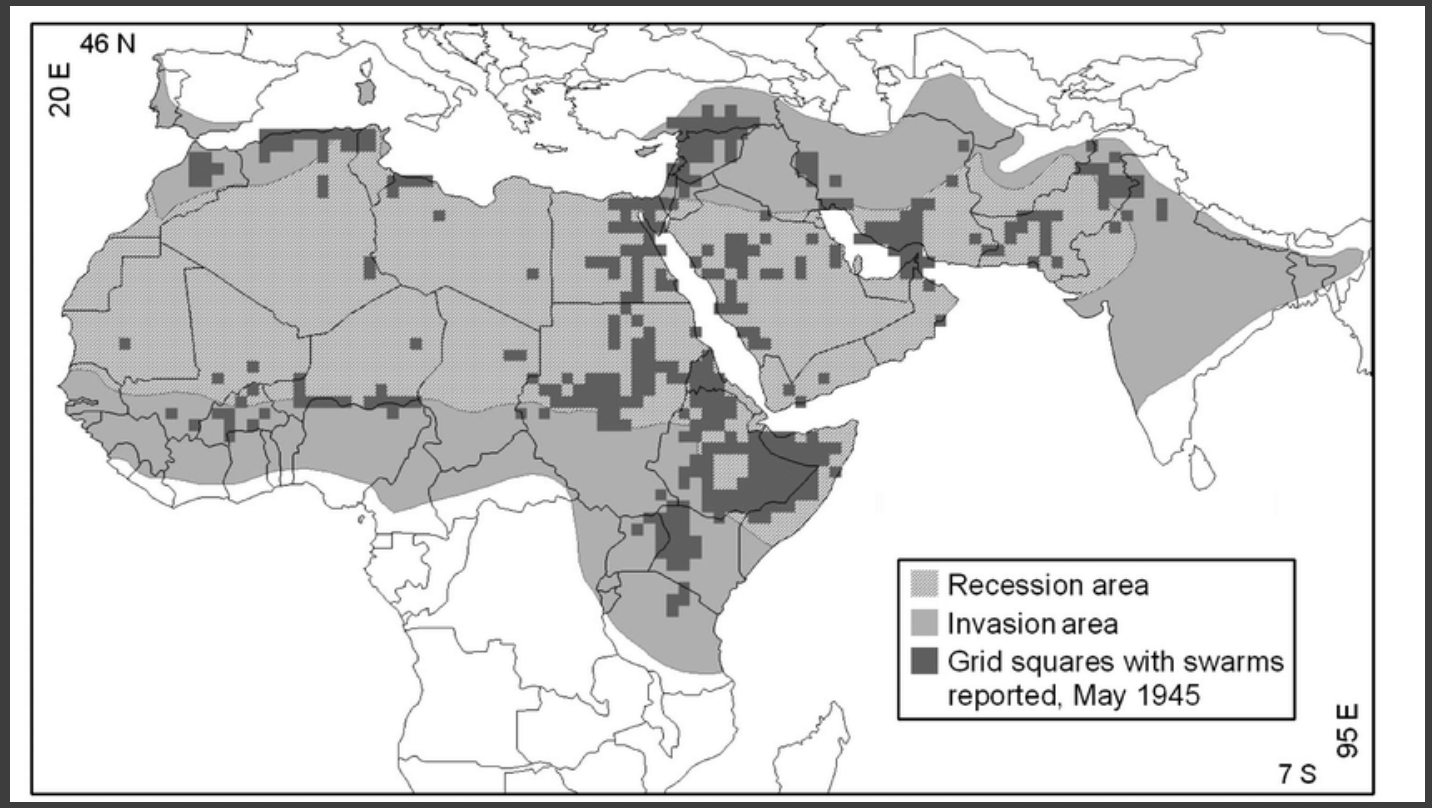
- Physical change due to environmental conditions
- Density-dependent in locusts
- Solitary, cryptic coloration → Gregarious, disruptive coloration

Solitarious Phase (Isolated)

Gregarious phase (Crowded)

THE DESERT LOCUST:

- *Schistocerca Gregaria* (Forskål).
- Located in the Middle East and Africa
- Known for biblical plagues
- Best studied species of Schistocerca Genus
- Occupies basal position of phylogeny
- Phase polyphenism in non-swarming locusts



WHY RAMAN? TRADITIONAL TECHNIQUES FOR ANALYSIS

Chemical Analysis:

- Focus: Determining molecular underpinnings of phase transitions.
- Method: Look for specific chemical differences between phases or observe genetic information of locust species.
- Studies may also explore evolutionary connections within the genus.
- Downfalls: Expensive and laborious procedures.

Behavioral Assays:

- Originally use a rectangular arena with two opposite chambers.
- One chamber contains a group of gregarious individuals, and the other is left empty.
- Specimen's behavior is observed and recorded.
- Downfalls: Lengthy process, requiring active observation from researchers.

MATERIALS AND METHODS

SAMPLE COLLECTION AND ANALYSIS

Collection

1. Grasshoppers raised

Raised in both isolated and crowded rearing conditions on a wheat diet

2. Hemolymph harvested

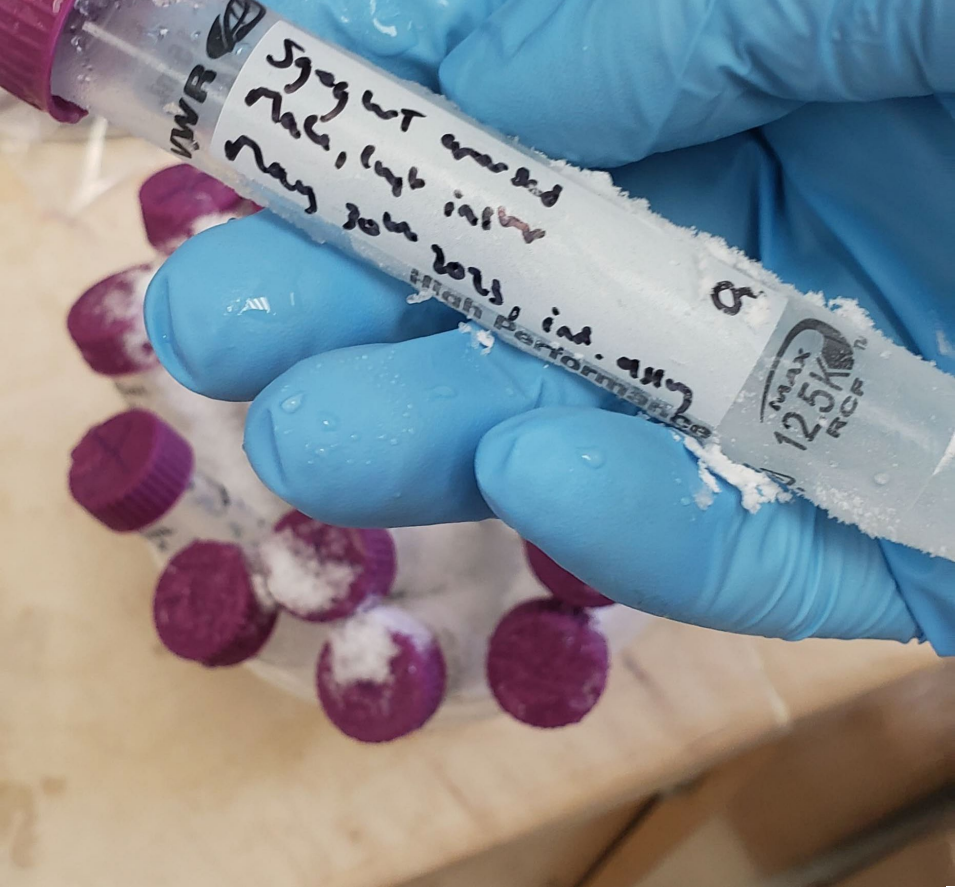
Marked for sex, conditions, diet, age, and species before storage

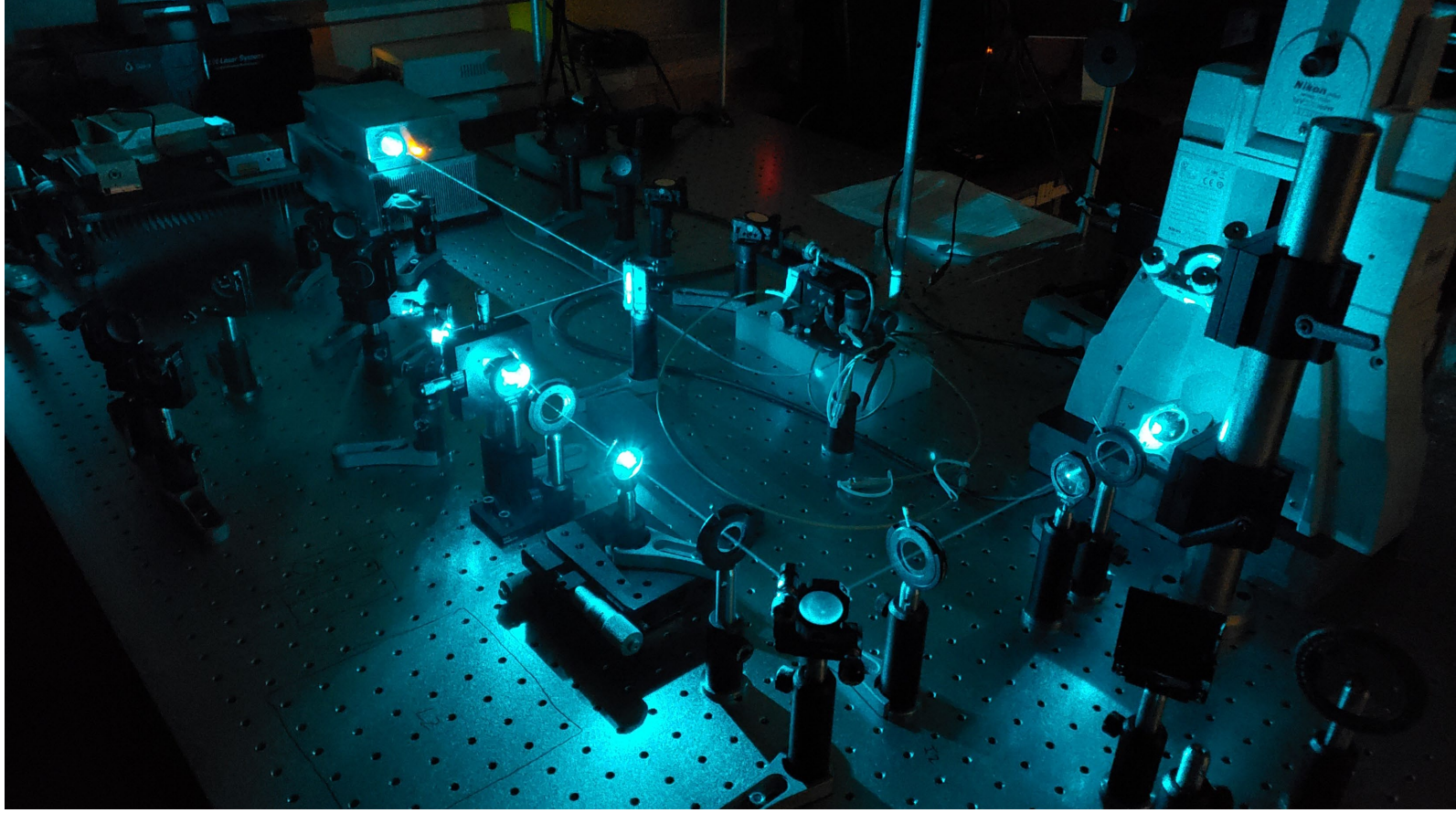
20 µL of hemolymph taken and was stored at -80 °C

Analysis

1. Scanned using a confocal Raman microscope with a 488nm laser with a power of 1mW and acquisition time of 1 second.
   - 25 spectra gathered per sample
   - 100 samples total, 2500 spectra

2. Spectra were uploaded to PLS_Toolbox in MATLAB for analysis and raw spectra were processed.
   - Partial least squares discriminant analysis (PLS-DA) was used to differentiate between crowded and isolated conditions.
   - Identified key peaks at specific wavenumbers: 1008, 1162, 1196, and 1530 cm^{-1}.
   - Key peaks analyzed by Kruskal-Wallis One-Way ANOVA at a significance threshold of 0.05.

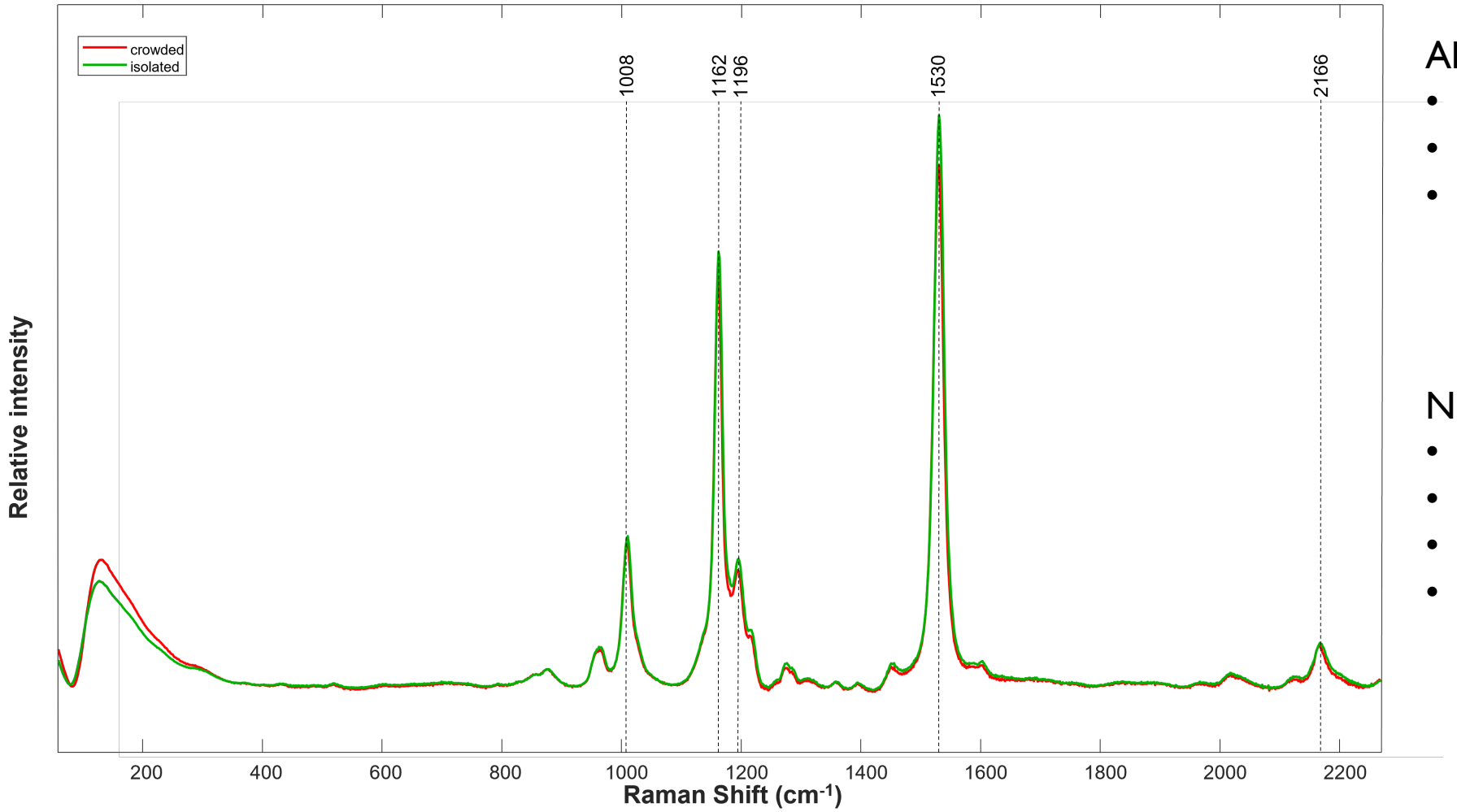




PICTURES!

RESULTS AND DISCUSSION

SPECTRA: CROWDED VS. ISOLATED



All spectra were:

- Averaged by group
- Baselined-corrected
- Normalized

Notable peaks

- 1008
- 1162
- 1530

} carotenoids

- 1196 phenolic compound

PLS-DA: PARTIAL LEAST SQUARES DISCRIMINANT ANALYSIS

```
CV RESULTS
Confusion Matrix (CV):
    Class:        TPR        FPR        TNR        FNR         N      Err        P          F1
  crowded       0.88167    0.10615    0.89385    0.11833    1200    0.11200    0.88462    0.88314
  isolated      0.89385    0.11833    0.88167    0.10615    1300    0.11200    0.89110    0.89247
Matthew's Correlation Coefficient =  0.776
```

PLS-DA discriminates based on differences in each spectrum

Reports

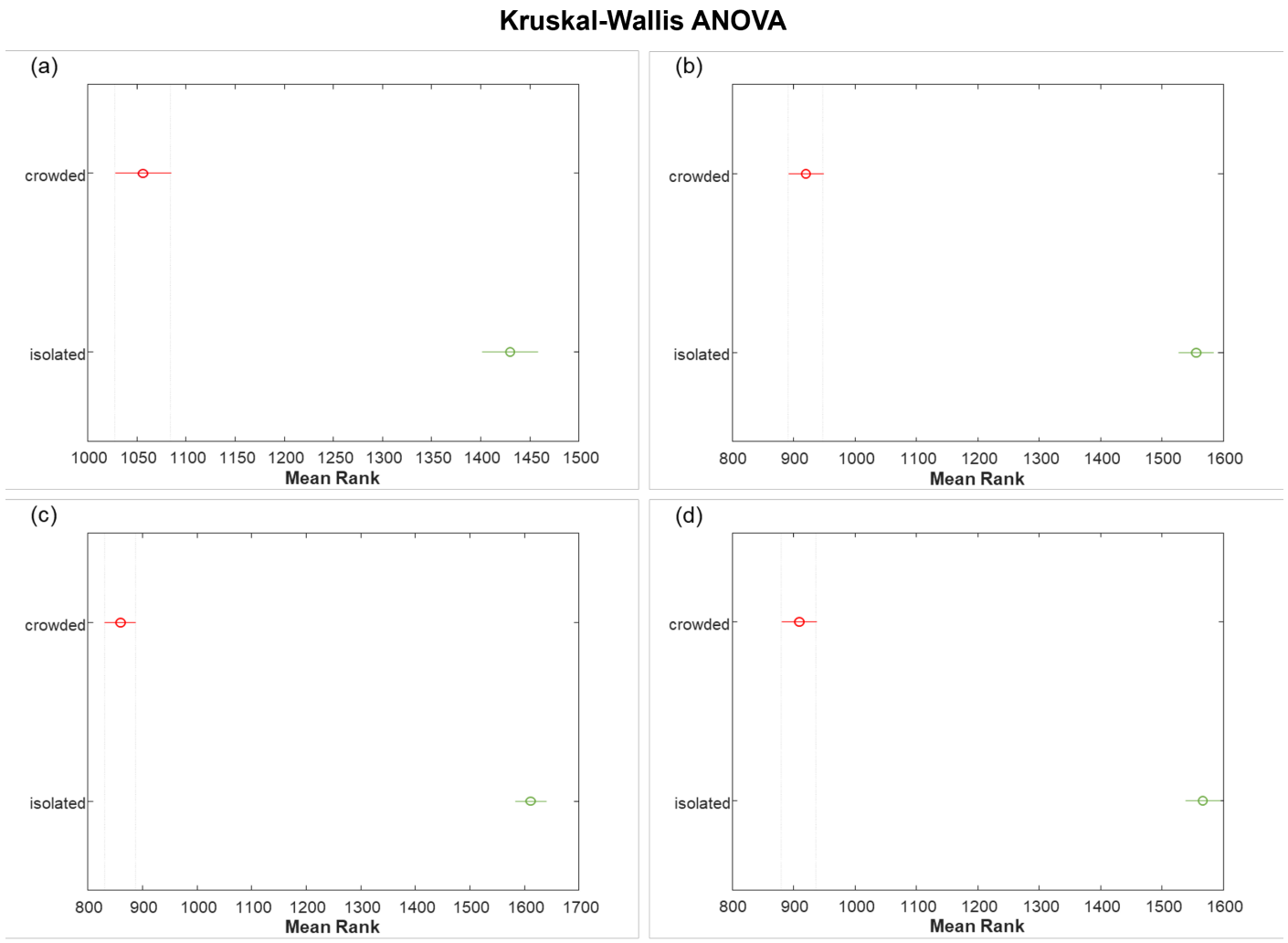
- TPR – true positive rate
- F1 – reports the ability of the model to accurately predict a class while minimizing false positives
- Matthew's correlation coefficient – reliability of the model, ranges from -1 to 1

KRUSKAL-WALLIS

- Nonparametric analysis
  - Normal distribution not required
- Mean intensities ranked

Peaks tested:

- (a) 1008 – isolated higher
- (b) 1162 – isolated higher
- (c) 1196 – isolated higher
- (d) 1530 – isolated higher



CONCLUSIONS

SUCCESS AND POTENTIAL FUTURE USE

Raman spectroscopy proves to be an effective analytical tool for detecting rearing density in locusts.

- Achieved high accuracy rates of 88.2% for crowded and 89.4% for isolated conditions.
- Significance observed in peaks at 1008, 1162, 1196, and 1530 cm^{-1} between isolated and crowded groups
  - This could be due to a difference in dietary conditions

Potential for Further Applications:

- Raman spectroscopy can be extended to analyze differing conditions relating to rearing density
- Specific subgroup conditions can provide valuable data beyond field applications.

THANK YOU!

Aidan Holman, Luke Osborne, and Axell Rodriguez for their help as other undergraduates of this program.

To Dr. Dmtiry Kurouski as the professor who advised me for this summer.

The CBTS and DHS officials and personnel who gave presentations and ran the program throughout the summer. Especially Dr. Heather Manley Lillibridge, Mr. Chris Scarmardo, and Dr. Mary Bryk.

Citrus greening disease identification, molecular diagnostics and development towards resistance against *C*Las infection

Maximiliano Hernandez
Cross-Border Threat Screening and Supply Chain Defense
Summer 2023

Outline:

- Background
- Objectives
- Citrus greening identification
- *Rhizobium*-mediated transformation
- Hairy root identification
- Hairy root diagnostics
- Conclusions
- Acknowledgements
- references

Background:

In the United States, Florida, Texas, California, and Arizona are responsible for the entire citrus industry with 42% of citrus cultivating from Florida as of 2019-20.

Global citrus cultivation

- The U.S. is the 3rd largest exporter of citrus following Brazil and China as of 2015.

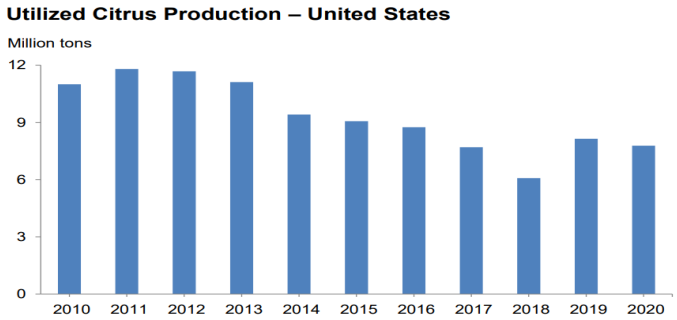
USA citrus cultivation

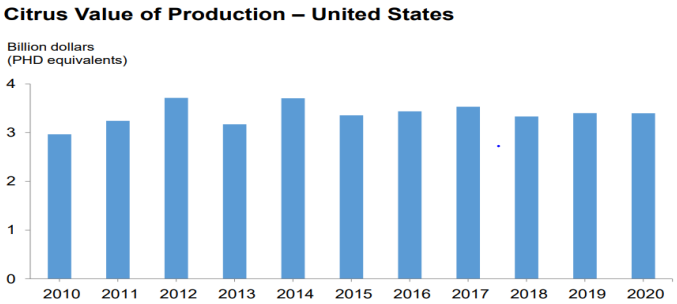
- 7.78 million tons of citrus was produced during 2019-20.
- Down 4% since 2010.

Global and National citrus production:

	Million MT					
	1999–2000	2004–05	2009–10	2011–12	2013–14	2015–16
Brazil	16,524	16,606	15,830	20,482	16,850	14,350
China	2,710	4,200	6,500	6,600	7,600	7,000
United States	11,894	8,293	7,478	8,166	6,153	5,371
Mexico	2,881	4,120	4,051	3,666	4,400	3,535
EU-27[a]	n/a	n/a	6,244	6,023	6,712	6,055
Spain[a]	2,710	2,700	n/a	n/a	n/a	n/a
Italy[a]	1,900	1,007	n/a	n/a	n/a	n/a
Egypt	1,730	1,750	2,401	2,350	2,570	2,750
Turkey	1,050	1,280	1,690	1,650	1,700	1,700
South Africa	1,047	1,120	1,459	1,466	1,620	1,560
Greece[a]	950	820	n/a	n/a	n/a	n/a
World total	43,196	45,434	49,151	53,830	51,008	45,763

Global sweet orange production 2000-2015 (2020).



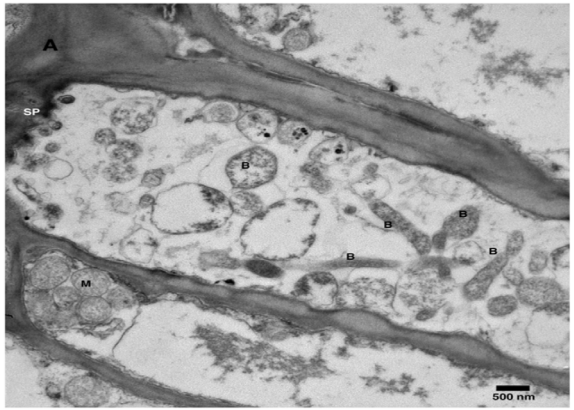


Citrus Fruits 2020 Summary (August 2020) 7
USDA, National Agricultural Statistics Service.

Background:

- Citrus greening disease: leading cause of farming pestilence in citrus species worldwide (Huanglongbing).
- *Candidatus Liberibacter asiaticus* (*C*Las): phloem limited fastidious gram-negative bacterium in the *Rhizobeaceae* family.
- Vectored by the asian citrus psyllid, *Diaphorina citri*.
- 2004: initial presence in Florida formally introducing the disease in the U.S.

Asian citrus psyllid and nymphs, UCIMP (2021)



*C*Las in phloem under an electron microscope, Hilf et al. (2013).

Background:

	(1)	(2)	(3)	(4)	(5)	(6)
Region	Surveys Count	Average Number Acres	Total Acres	Average % Infected acres	Average % Infected Trees	Average % Yield Loss
Central	44	693	28,414	88	83	45
Central/SW	11	1,962	17,655	90	74	37
Southwest	21	5,818	110,545	92	77	33

Citrus industry responses, University of Florida (updated 2023)

- Profit loss due to *C*Las measured within $3.36 billion as of 2006 according to the University of Florida.

Objectives:

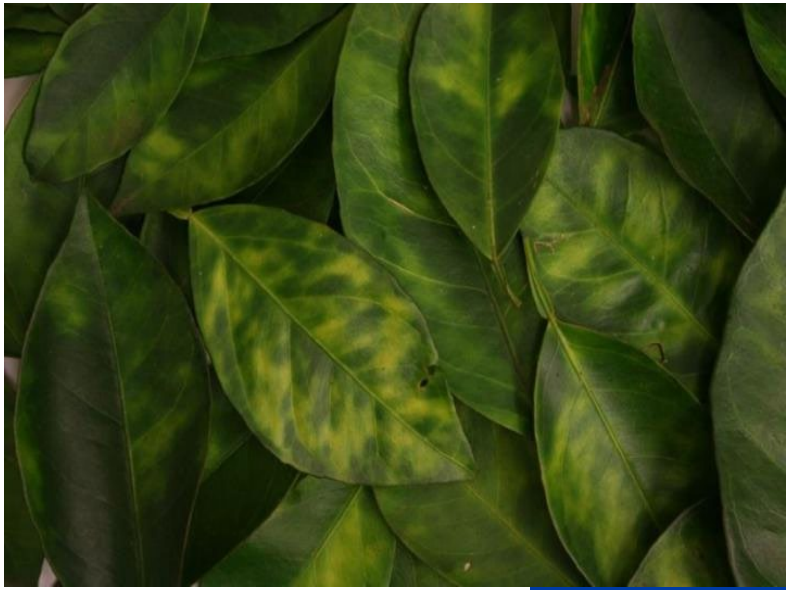
- Use of identification and diagnostic methods to screen for resistances and enhance prevention and treatment against citrus greening disease.
- To understand the functions of current tools used against citrus greening disease.

Citrus greenhouse conditions at A&M agrilife extension at Weslaco (2023).

Citrus greening identification methods:

Common visual expressions of citrus greening

- Blockage in the phloem tissue due to movement of bacteria causes hindrance in nutrient flow leading to discoloration and leaf loss.
- Uneven fruit size, lopsidedness and loss of preferable fruit flavor.
- Irregular new leaf formation with a characteristic notch along with leaf twisting and curling.



"Blotchy mottle" appearance due to HLB, University of Florida (2023).

Notch presence due to HLB exposure.

Severe warpage of new leaf due to long persistent HLB exposure.

Rhizobium-mediated transformation:

Hairy Root induction

- To combat the unculturable nature of *C*Las, the induction of hairy roots coupled with an intact xylem and phloem allow for propagation.
- Hairy roots are induced by contact of the *Rhizobium rhizogenes* bacterium that introduces its root-inducing transfer DNA (Ri T-DNA) into plant cells
- Multiple encoded root locus (ROL) genes included convert shoot cells into root cells through formation of tumor-like tissue.

Citrus plant with hairy root evidence.

Callus formation as a result of ROL gene transformation

Rhizobium-mediated transformation:

*C*Las positive branches in vermiculite soil inoculated with R. Rhizogenes.

Rhizobium inoculated petioles in vermiculite soil.

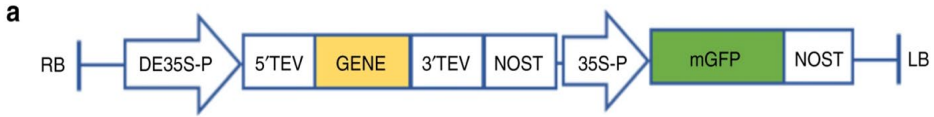
Methods of inoculation

- *C*Las positive branches from previous HLB-graft-infected trees can be trimmed for inoculation.
- Petioles that attach the leaf to the branch can also be inoculated as plant cells have high cell plasticity.

Hairy root identification:

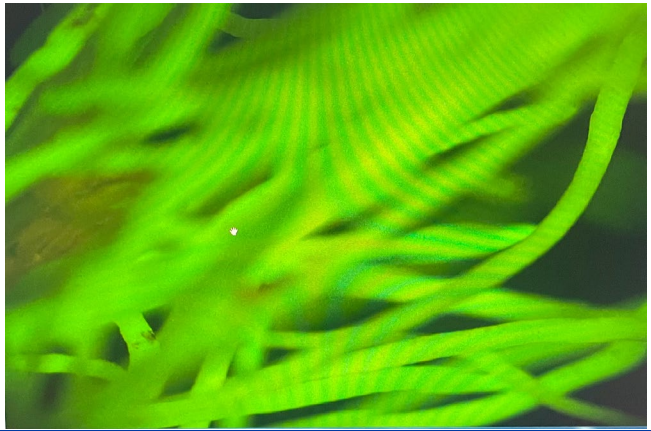
Green Fluorescence Protein overexpression as a marker

GFP overexpression can be used to confirm for subsequent transgenic material.



typical transgene overexpression construct with a gene and a GFP marker cassette, Irigoyen et al. (2020)

Non-transgenic root formation visualized using fluorescence microscopy.

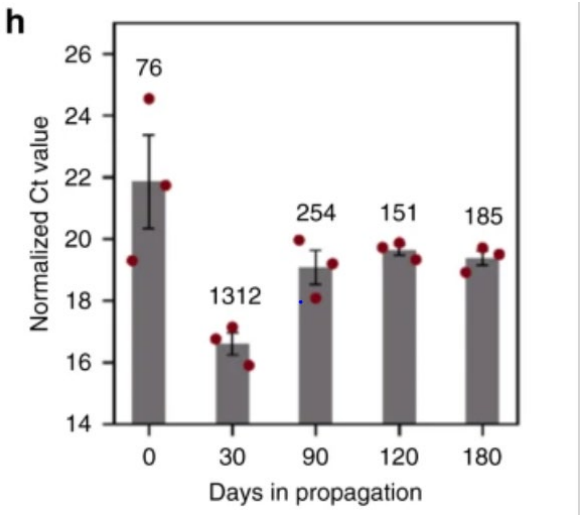


GFP expression of transgenic hairy root formation using fluorescence microscopy.

Hairy root diagnostics

PCR-based diagnostic tools

- PCR amplification of either the GFP or rolB/rolC genes encoded on the binary and Ri T-DNA plasmids are used to confirm hairy root gene expression.
- The PCR control genes used for citrus plants is GAPC2 endogenous genes.
- *C*Las estimation is conducted using specific markers for ribosomal protein and ribonucleotide reductase β-subunit genes.



qPCR quantification between healthy and *C*Las infected samples example, Irigoyen et al., (2020).

Conclusions:

Significance of hairy root gene expression

- *R. rhizogenes*-mediated hairy root induction beneficial towards bio-engineering and gene editing applications against *C*Las.
- Identification and quantification measures using visual expressions and molecular diagnostics is beneficial when comparing different plant constructs.
- Potential antimicrobial agents and gene expressions resistant to *C*Las infection using quantifiable data can pave the way for solving difficulties with other incurable pathogens.

Acknowledgements:

This material is based upon work supported by the U.S. Department of Homeland Security under Grant Award Number 18STCBT00001. I would Like to thank Dr. Kranthi Mandadi, Rajitha Kavuri, Aditya Kulshreshtha and Victoria Mora for their assistance throughout this project.

CROSS-BORDER THREAT SCREENING AND SUPPLY CHAIN DEFENSE

A Department of Homeland Security Center of Excellence

References:

Asian Citrus Psyllid and Huanglongbing Disease Management Guidelines--UC IPM. (2021). UC IPM. https://ipm.ucanr.edu/PMG/PESTNOTES/pn74155.html

FE983/FE983: Impact of Citrus Greening on Citrus Operations in Florida. (updated 2023). Ask IFAS. https://edis.ifas.ufl.edu/publication/FE983

Hilf, M. E., Sims, K. R., Folimonova, S. Y., & Achor, D. S. (2013). Visualization of 'Candidatus Liberibacter asiaticus' cells in the vascular bundle of citrus seed coats with fluorescence in situ hybridization and transmission electron microscopy. Phytopathology, 103(6), 545–554. https://doi.org/10.1094/PHYTO-09-12-0226-R

Irigoyen, Ramasamy, M., Pant, S., Niraula, P., Bedre, R., Gurung, M., Rossi, D., Laughlin, C., Gorman, Z., Achor, D., Levy, A., Kolomiets, M. V., Sétamou, M., Badillo-Vargas, I. E., Avila, C. A., Irey, M. S., & Mandadi, K. K. (2020). Plant hairy roots enable high throughput identification of antimicrobials against Candidatus Liberibacter spp. Nature Communications, 11(1), 5802–5802. https://doi.org/10.1038/s41467-020-19631-x

Talon, Caruso, M., & Gmitter jr., F. G. (2020). Global economics and marketing of citrus products. In The Genus Citrus. Elsevier Science & Technology. https://doi.org/10.1016/B978-0-12-812163-4.00023-1

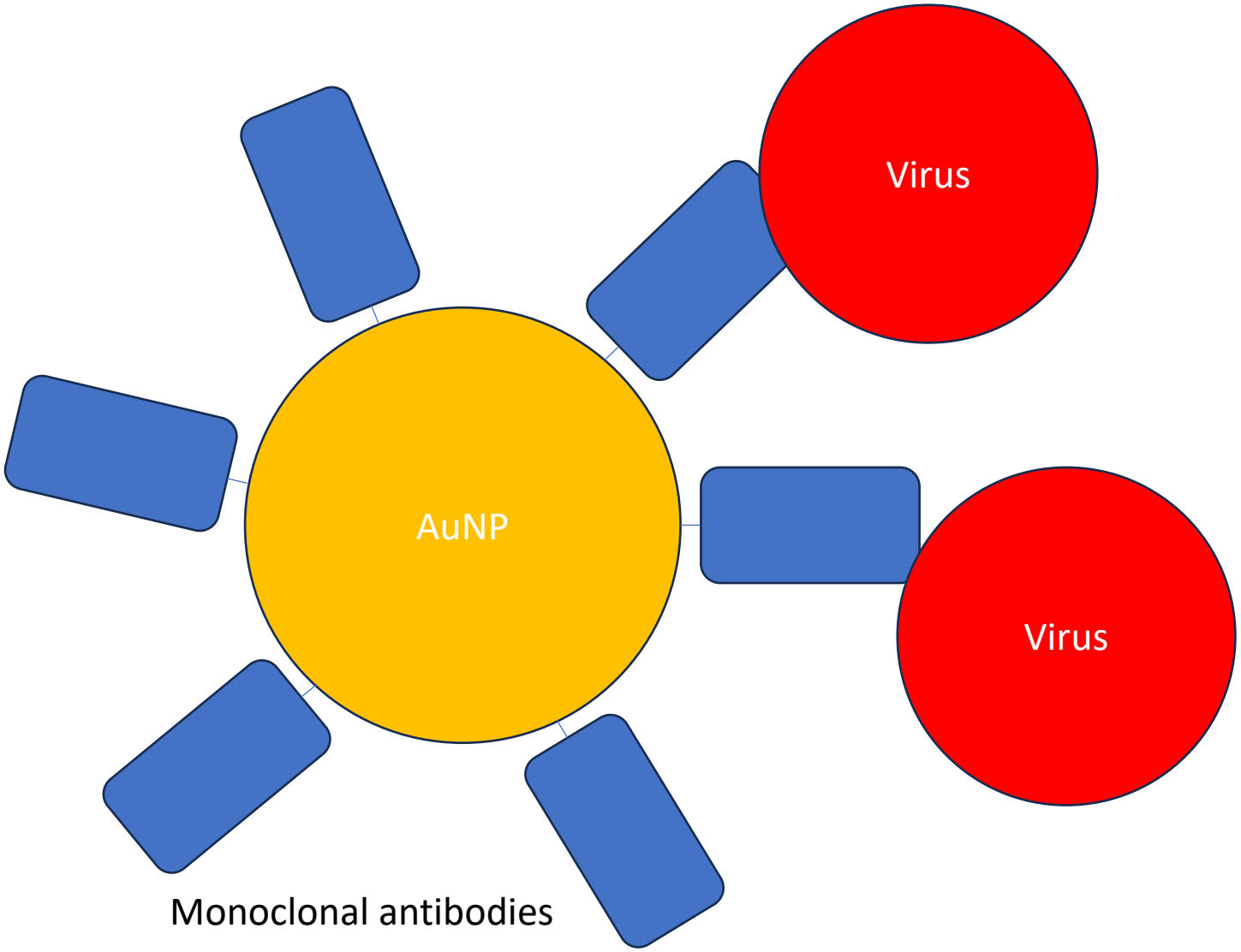
Utilization of Atomic Force Microscopy-Infrared Spectroscopy to Access Secondary Structure and Stability of Proteins

Axell Rodriguez

Kurouski Lab

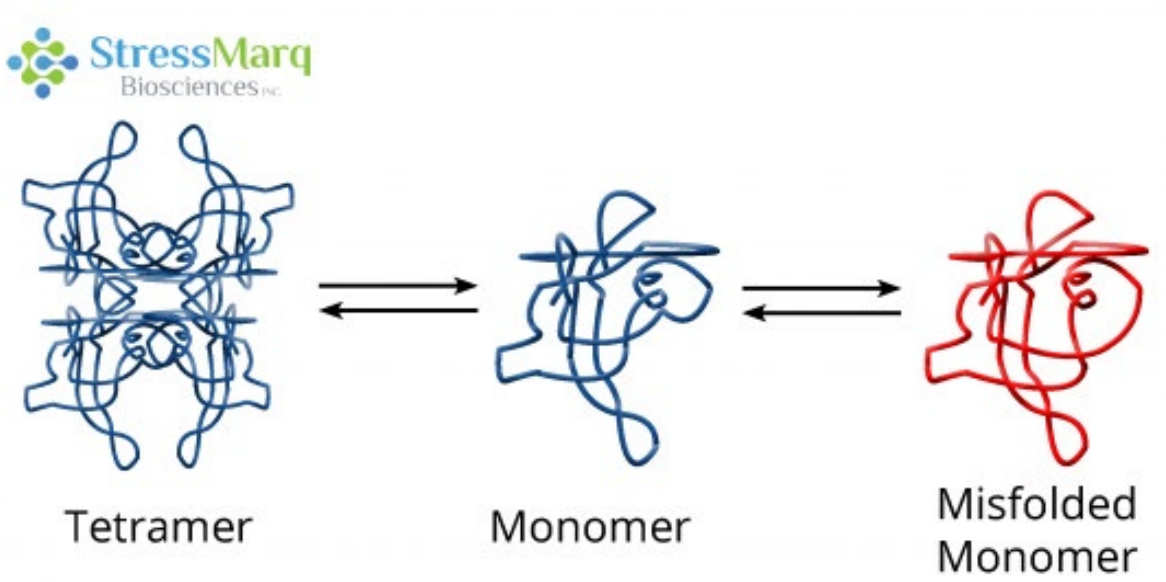
CBTS-DHS

Proteins as sensors of pathogens



Transthyretin (TTR) as a model protein system

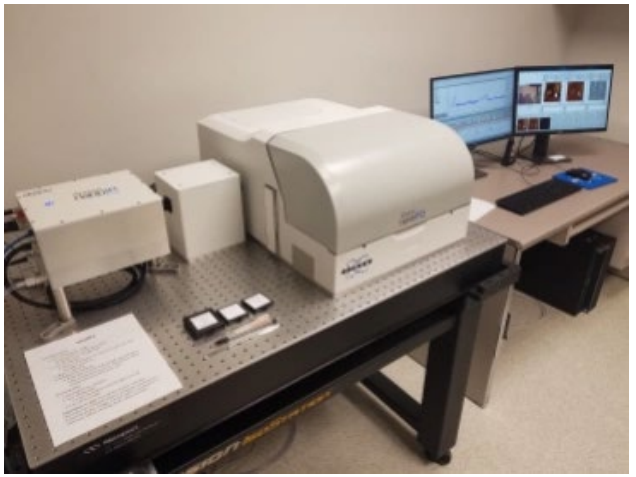
- Transports thyroxine (T4) & Retinol-binding protein (RBP)
- Model protein to characterize changes to structure and stability

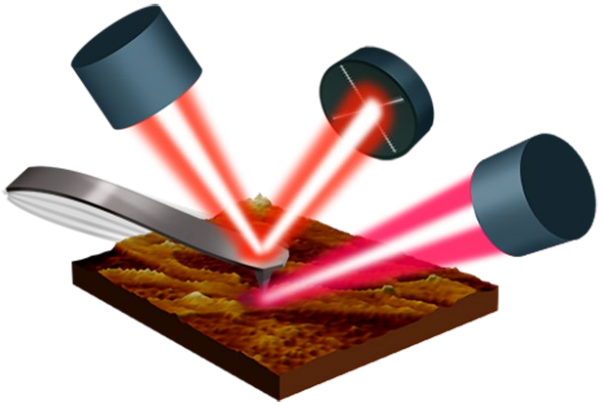


Thomson, P. (2019). Transthyretin (TTR) In Amyloid Diseases, *Scientific Review.*

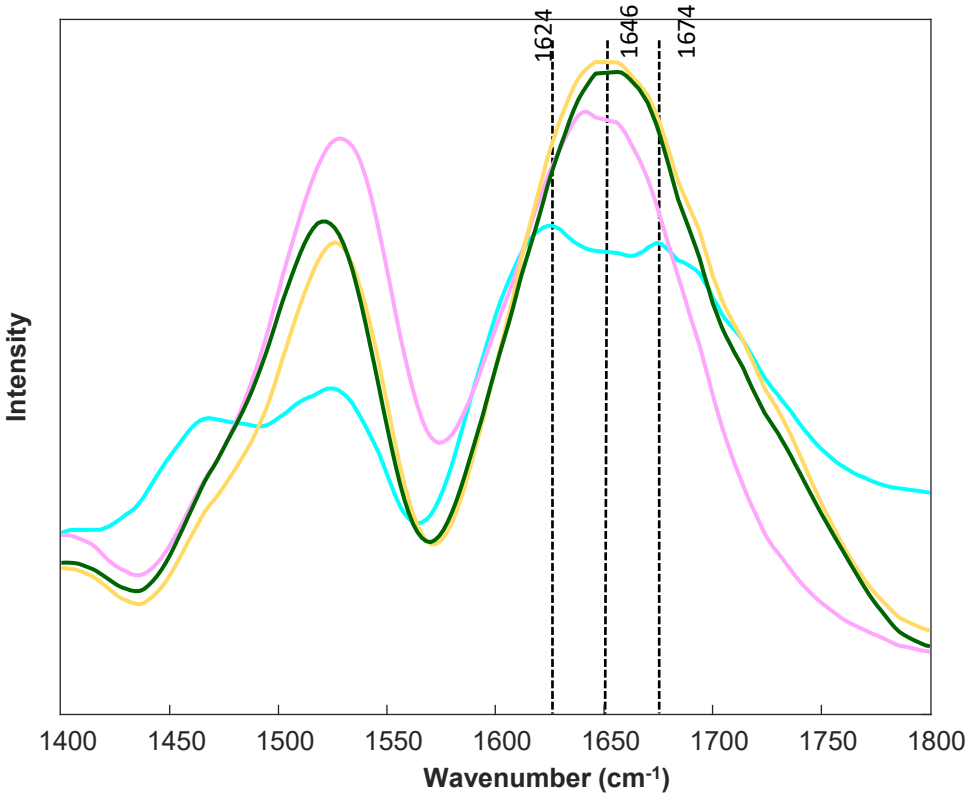
Nano-IR

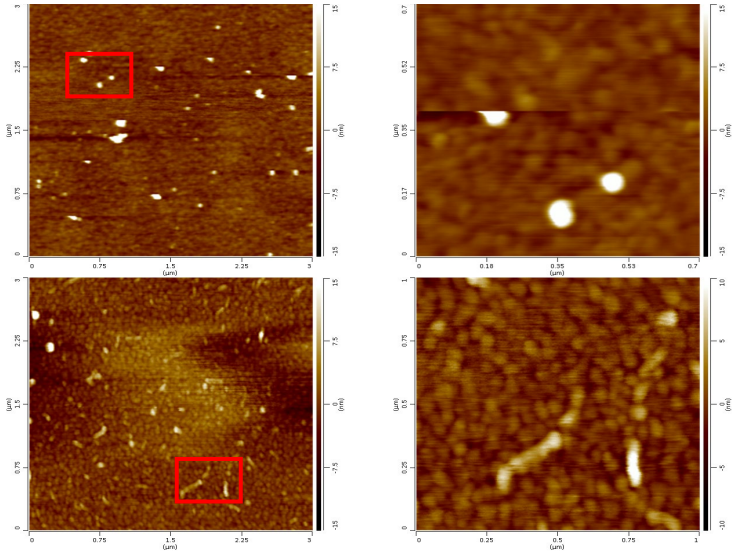
- Direct detection for pathogens
- Idea to create antibodies using nanoparticles to target pathogen
- Allows characterization of secondary structure



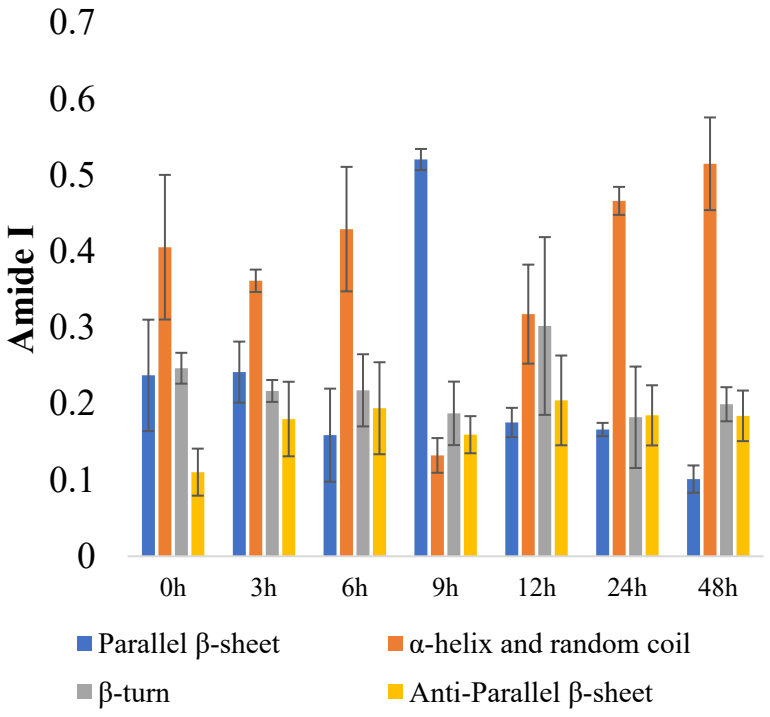


Data Acquisition

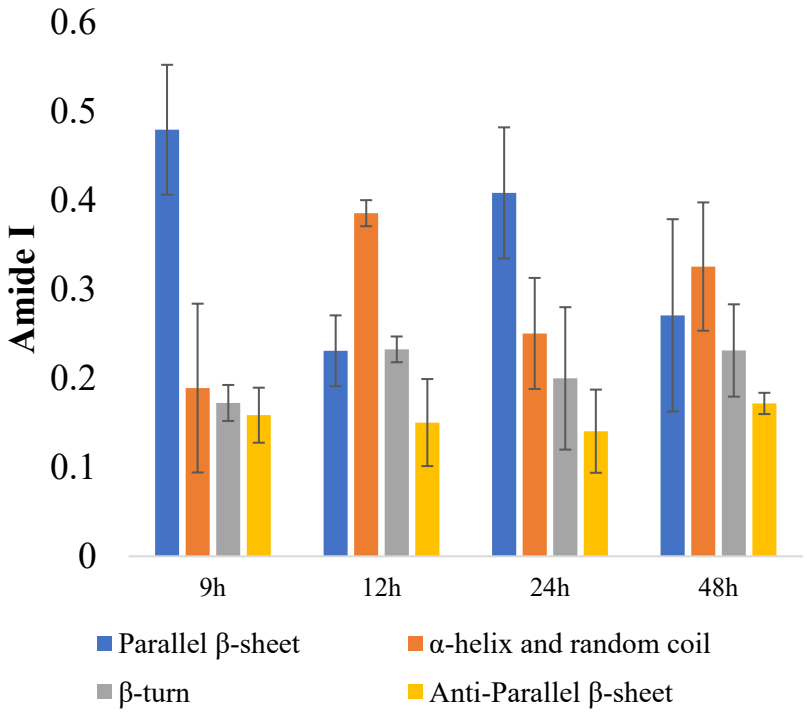




Data Results



Oligomers



Fibrils

Raman Spectroscopy

- Indirect detection of pathogens using Raman Spectroscopy
- Rice stress through different levels of arsenic
- Alteration to the project: grow hydroponically

Conclusion

- Learned two methods that can be used to detect pathogens, both direct (Nano-IR) & indirect (Raman Spectroscopy).

- Improved working with peers, writing, & presentation.

Temporal infection analysis of TMV in Nicotiana benthamiana

Jocelyn Gutierrez

Cross-Border Threat Screening and Supply Chain Defense

Summer 2023

Outline

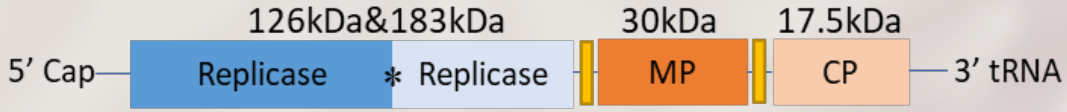
- Introduction
  - TMV and N. benthamiana
- Objectives
- Materials and Methods
  - Plant Materials
  - Rub-Inoculation of N. benthamiana
  - RNA Extraction
  - cDNA Synthesis
  - RT-PCR
- Results
  - Identification of TMV in N. Benthamiana
  - Molecular analysis of TMV in N. benthamiana
- Conclusions

Introduction

Tobacco Mosaic Virus (TMV)

- TMV – positive ssRNA virus
- Common viral disease that has a wide host range.
- Highly infectious virus that can cause significant damage to plants.
- Symptoms include discoloration (mosaic), wilting, and necrosis.

Schematic of TMV genome



TMV symptoms in Nicotiana benthamiana

Nicotiana benthamiana

- Tobacco species known to be a model plant for its efficiency for successful experiments.

- Key plant species for scientific research because of its ease of use.

- Using N. benthamiana to study TMV is that the plant is very susceptible to infection

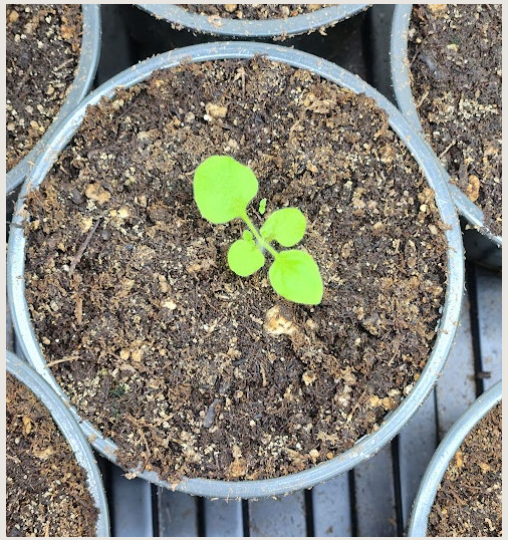
Objectives

1. Observe physiological symptoms of TMV in N. benthamiana at different time points.
2. Molecular characterization of TMV in N. benthamiana.

Materials and Methods

Plant Material

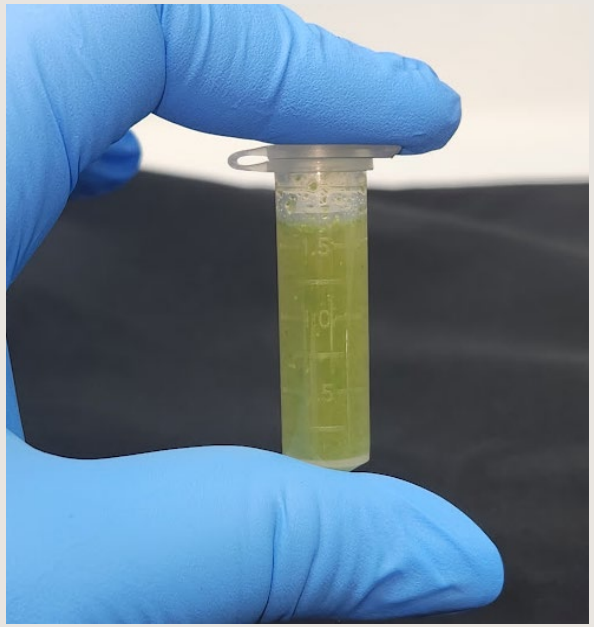
Seeds and soil used for germination



Germination after 1 week

N. benthamiana at 4 weeks

Rub-Inoculation of N. benthamiana



Previously infected tissue at 7 DPI mixed with inoculation buffer

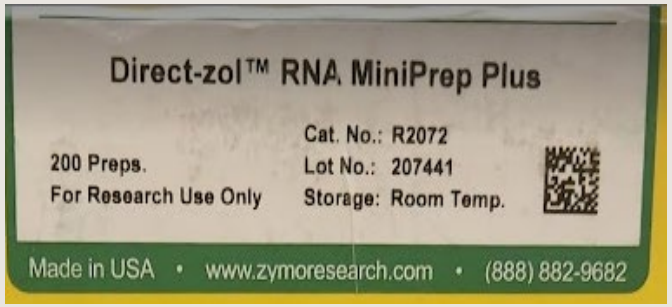
250 µL of the infected tissue buffer

Rub-inoculation of leaves

RNA Extraction

Grounding of tissue

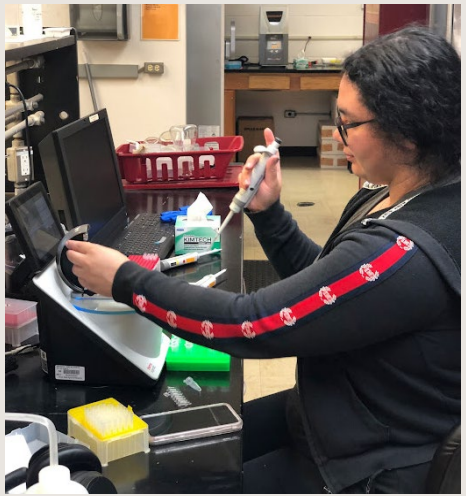
RNA extraction Kit



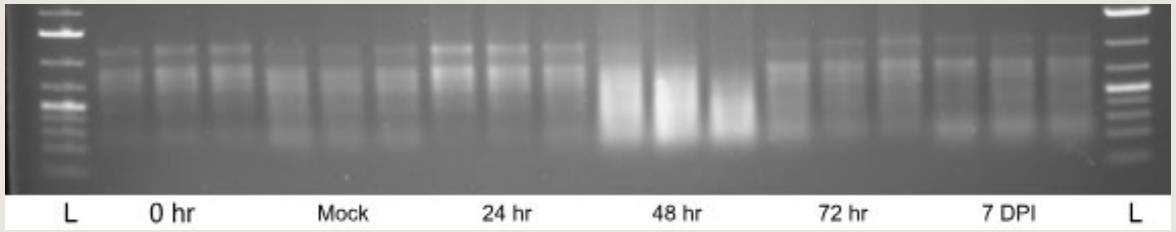
Trizol reagent

Ongoing extraction

RNA Quantification

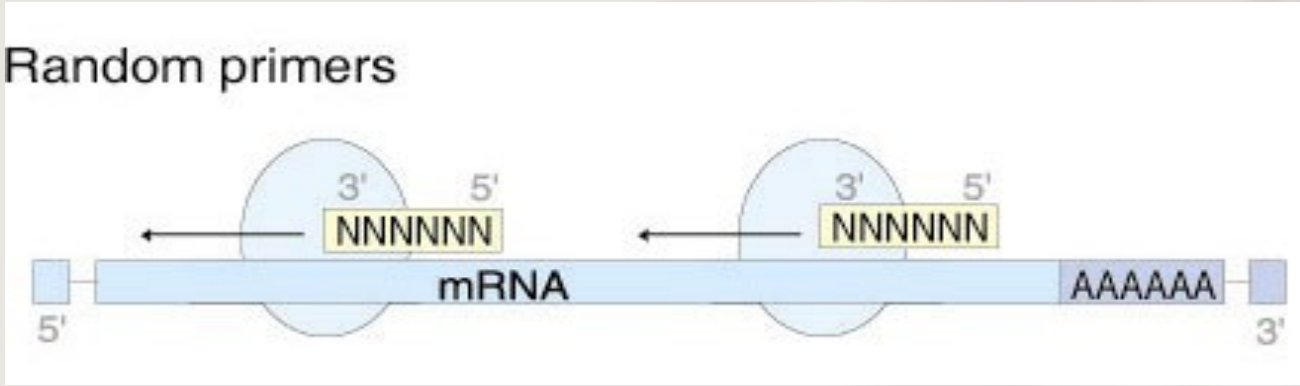


RNA Gel

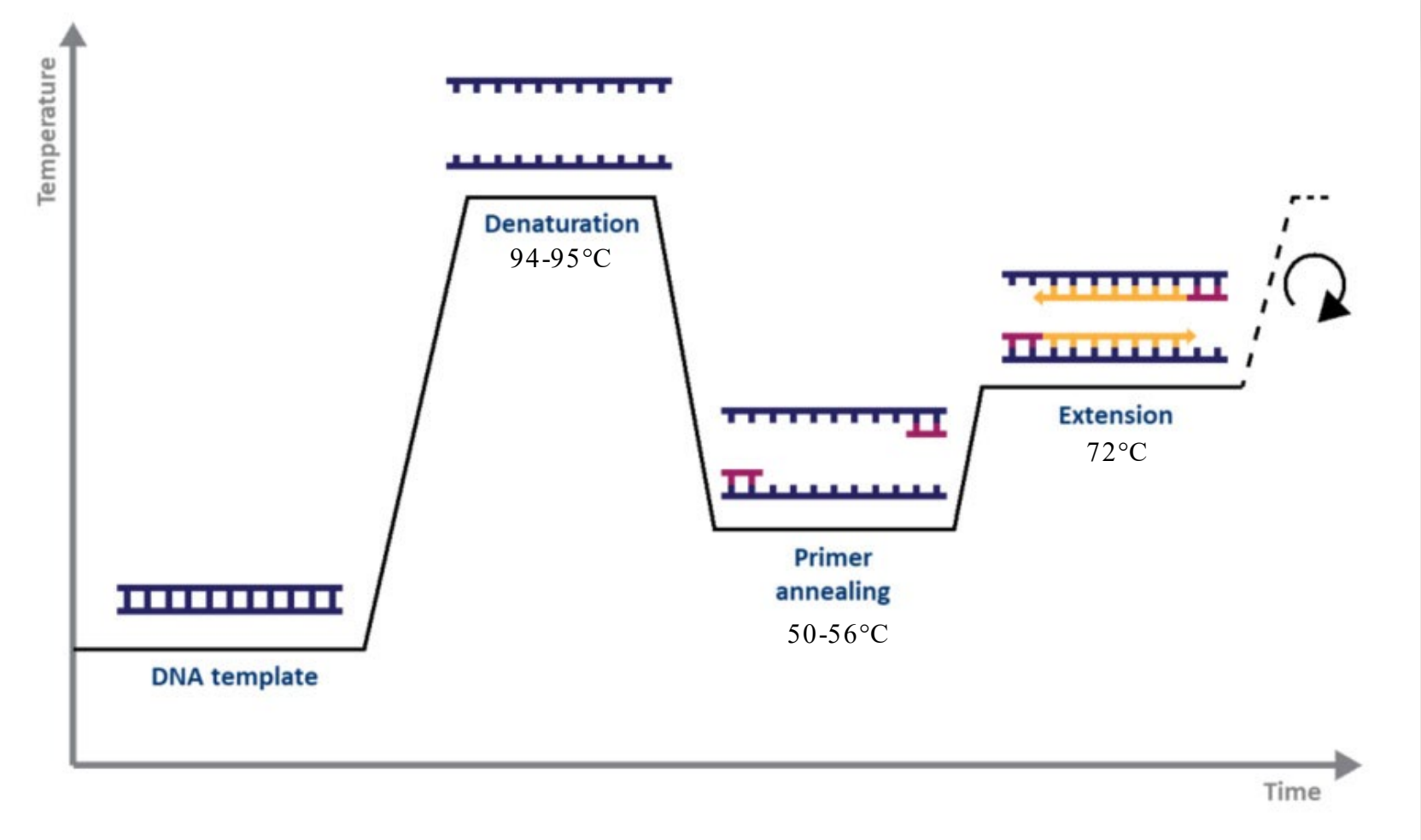


cDNA Synthesis

- RNA will be annealed using a random hexamer
- Gives random coverage to all regions of the RNA
- RNA is then reverse transcribed to cDNA pool by reverse transcriptase (RT), generating various lengths of cDNA.



Polymerase Chain Reaction



Results

Identification of TMV in N. benthamiana

N. benthamiana at 24 hours

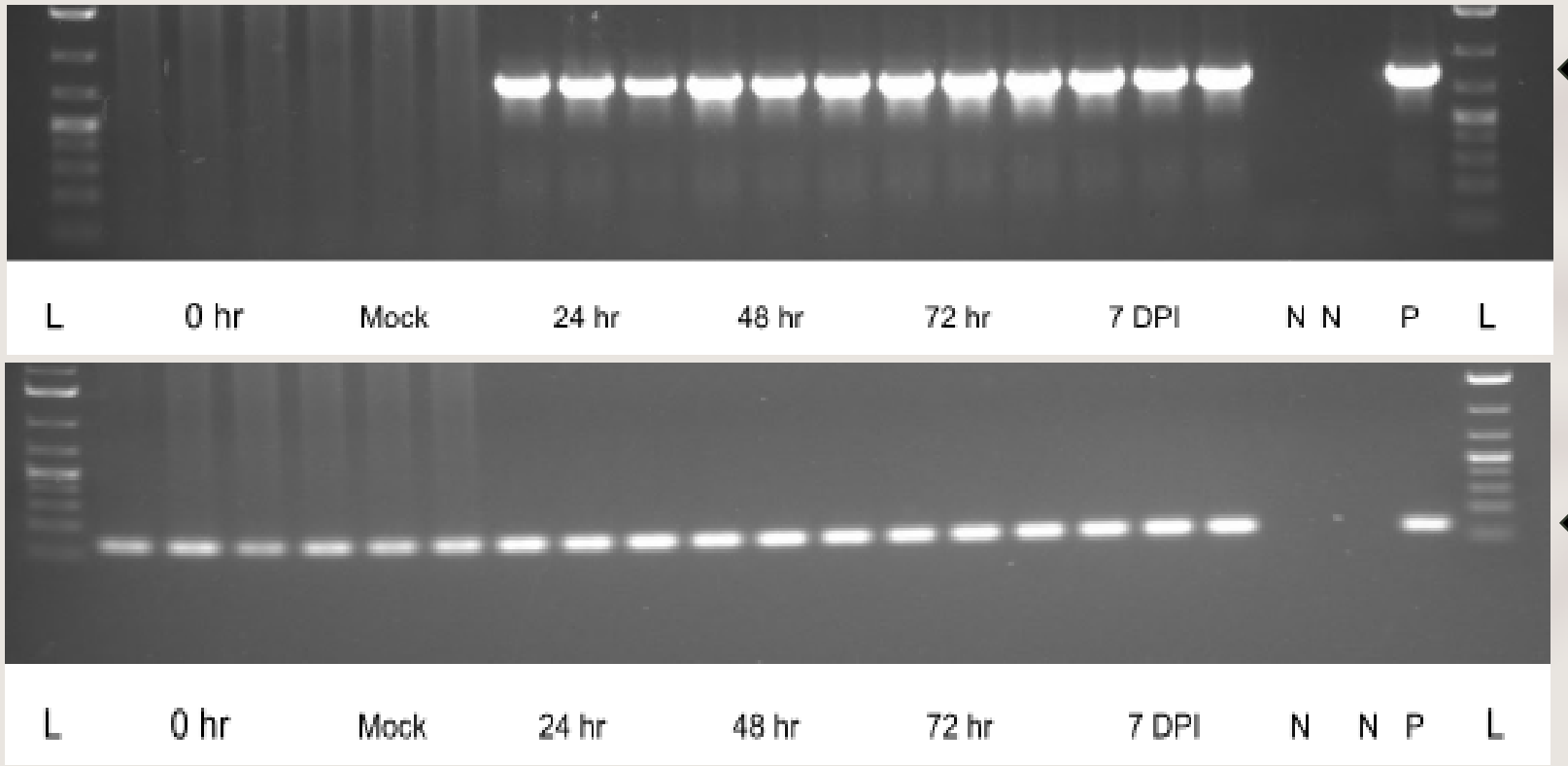
N. benthamiana at 48 hours

Identification of TMV in N. benthamiana

N. benthamiana at 72 hours

N. benthamiana at 7 DPI

Molecular Analysis of TMV in N. benthamiana



RT-PCR of TMV infected N. benthamiana samples at different time points

Conclusions

- Various temporal patterns emerge, influencing the severity and duration of symptoms.
- Understanding these temporal dynamics is crucial for developing effective strategies to control viral diseases in crops and protect agricultural yields.
- N. benthamiana provides a valuable model system for studying other plant viruses and their interactions with host plants.

Acknowledgements

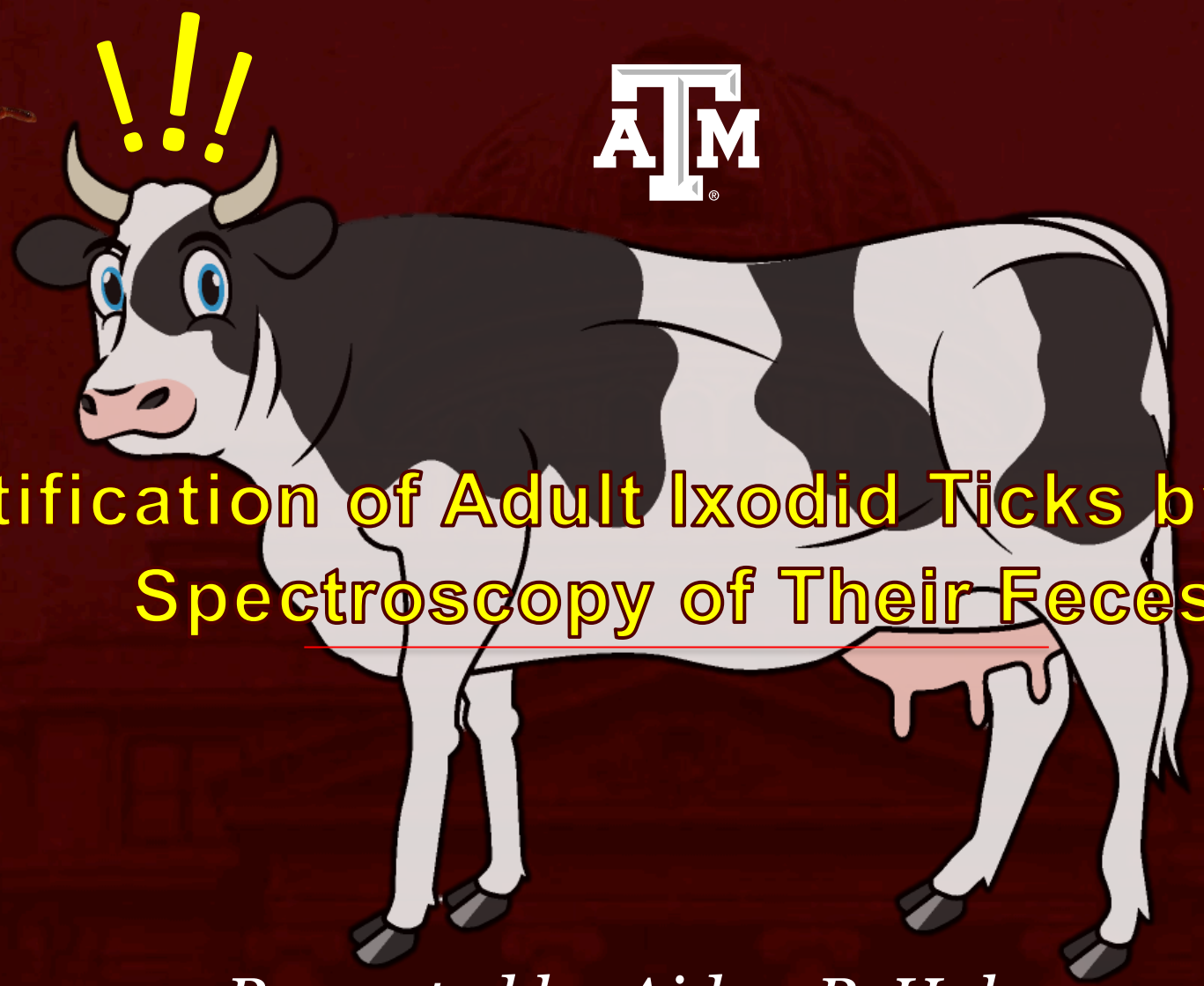
This material is based upon work supported by the U.S. Department of Homeland Security under Grant Award Number 18STCBT00001. I would like to thank Dr. Kranthi Mandadi, Rajitha Kavuri, Victoria Mora, and Aditya Kulshreshtha for their assistance throughout this project.

CROSS-BORDER THREAT SCREENING AND SUPPLY CHAIN DEFENSE

A Department of Homeland Security Center of Excellence

Questions?

Identification of Adult Ixodid Ticks by Raman Spectroscopy of Their Feces



Presented by Aidan P. Holman

CROSS-BORDER THREAT SCREENING AND SUPPLY CHAIN DEFENSE

A Department of Homeland Security Center of Excellence

BACKGROUND

Background

- Tick surveillance is crucial for controlling tick-borne diseases in cattle production.
- The US Cattle Fever Tick Eradication Program inspects cattle for tick presence.
  - Tick sizes can be too small to feel (Palmer et al. 1976).
- Tick-borne diseases, such as Babesiosis (Cattle Fever), pose a significant threat to cattle health and production.
  - Cattle Fever tick outbreak predicted to cost over $1.2 billion dollars within a year of surfacing (Anderson et al. 2010).
- Raman spectroscopy (RS) shows promise in identifying tick species and their feces.
  - RS is the photometric measurement of inelastically scattered photons from specimens under visible light.

METHODS

Methods

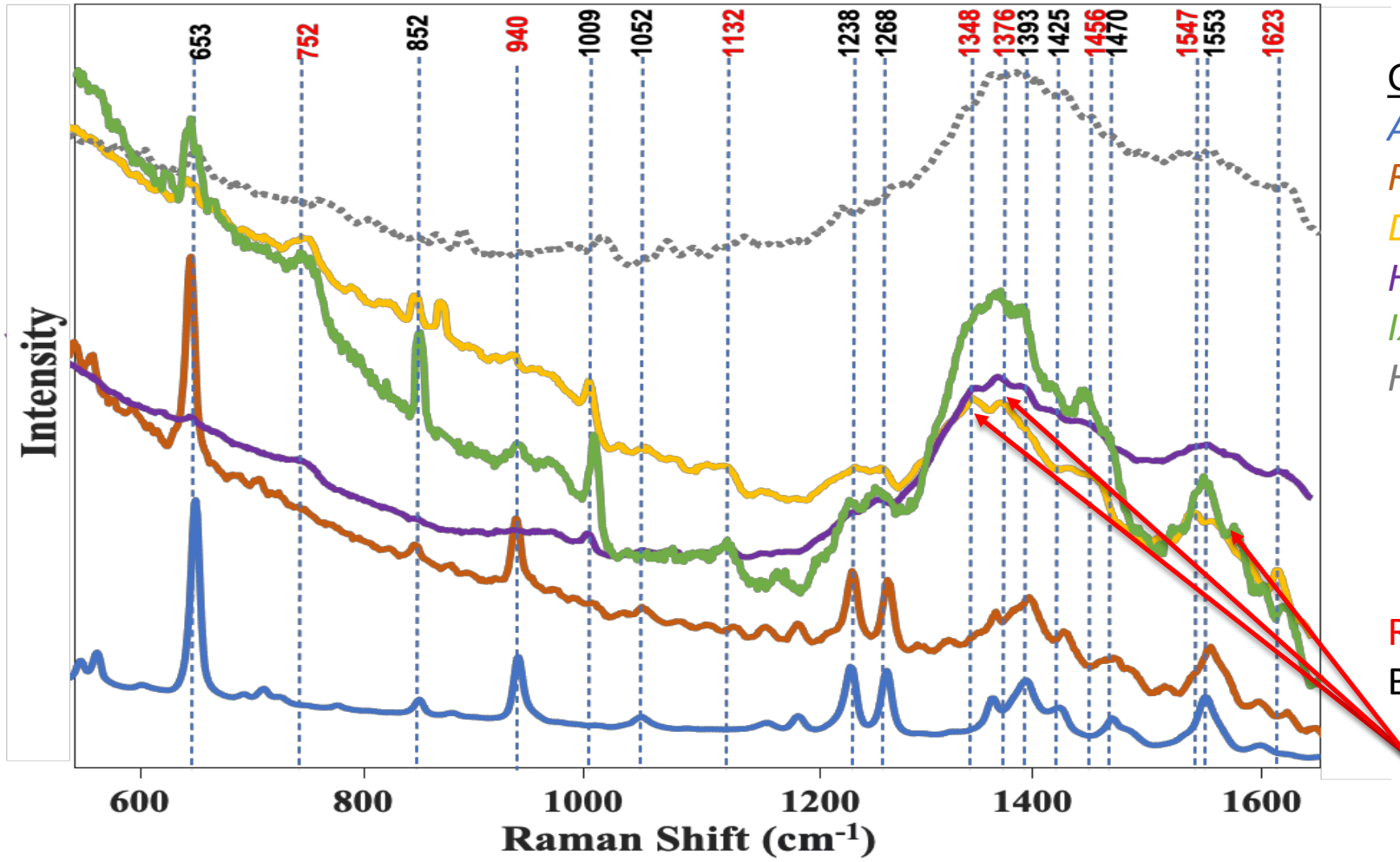
- We scanned 57 samples of ticks from multiple genera (*Amblyomma*, *Rhipicephalus*, *Dermacentor*, *Haemaphysalis*, and *Ixodes*) and 12 species and 2 samples of horn flies.
- PLS-DA is a discriminant analysis used to test the feasibility of a model to accurately identify individual spectra of the correct class.

	Actual Oranges (n=25)	**Actual Apples** (n=25)
Predicted as Oranges	20	2
Predicted as Apples	5	23
TPR	80%	92%

- Tick frass was collected from the skin of cattle and sheep from institutions in OK, WA, and several more in TX.
- Utilized a confocal Raman microscope with a 750nm laser and acquired 20-25 spectra from each fecal sample between 10-25 second acquisitions.

RESULTS

Results



Genus-level comparison:

Amblyomma spp.

Rhipicephalus spp.

Dermacentor spp.

Haemaphysalis longicornis

Ixodes scapularis

Haematobia irritans

Red bands = Blood

Black bands = Guanine

Heme group

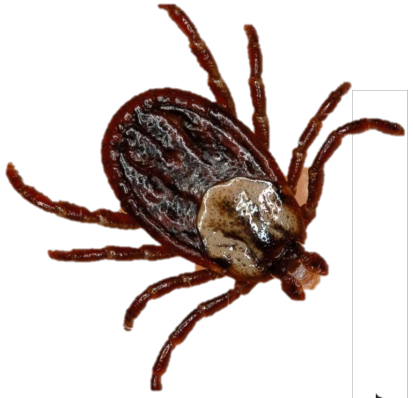
Results

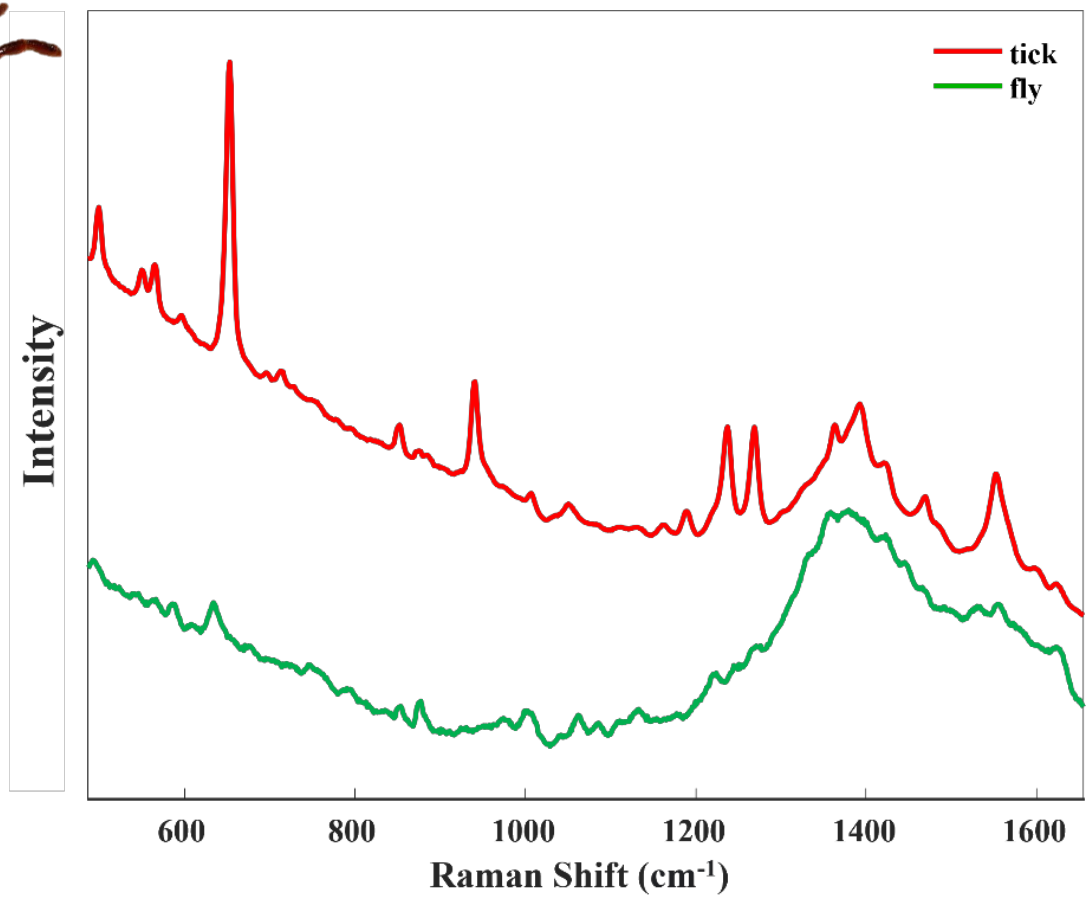
	Amblyomma (n=471)	***Rhipicephalus*** (n=59)	***Dermacentor*** (n=339)	***Haemaphysalis*** (n=80)	***Ixodes*** (n=20)
Predicted as *Amblyomma*	471	0	2	0	0
Predicted as *Rhipicephalus*	0	58	19	0	1
Predicted as *Dermacentor*	0	0	317	0	0
Predicted as *Haemaphysalis*	0	0	0	80	0
Predicted as *Ixodes*	0	1	1	0	19
TPR%	**100**	**98.35**	**93.5**	**100**	**95**

Results

	R. annulatus (n=18)	***R. microplus*** (n=21)	***R. sanguineus*** (n=20)
Predicted as *R. annulatus*	18	0	0
Predicted as *R. microplus*	0	20	0
Predicted as *R. sanguineus*	0	1	20
TPR%	**100**	**95.23**	**100**
	D. albipictus (n=180)	***D. andersoni*** (n=184)	***D. variabilis*** (n=20)
Predicted as *D. albipictus*	180	1	0
Predicted as *D. andersoni*	0	183	0
Predicted as *D. variabilis*	0	0	20
TPR%	**100**	**99.28**	**100**

Results





	Tick (n=969)	**Fly** (n=40)
Predicted as tick frass	954	0
Predicted as fly frass	15	40
TPR%	**98.45**	**100**

DISCUSSION/CONCLUSION

Discussion/Conclusions

- Raman enabled accurate identification of multiple genera (>93%) and species (>95%).
- Raman enabled accurate differentiation between ticks and horn flies (>98%)
- Horny fly feces were not similar to blood-like tick feces
  - Possible Explanation: A female D. andersoni tick can engorge up to 4,000 mg of blood in one sitting while a female horn fly will imbibe 1.71 mg per feeding event (Annand 1941, Kaufman and Phillips 1973).
  - Additionally, their digestive processes are different, e.g. insects primary waste product is uric acid and ticks primary waste product is guanine (Sonenshine and Roe 2014).

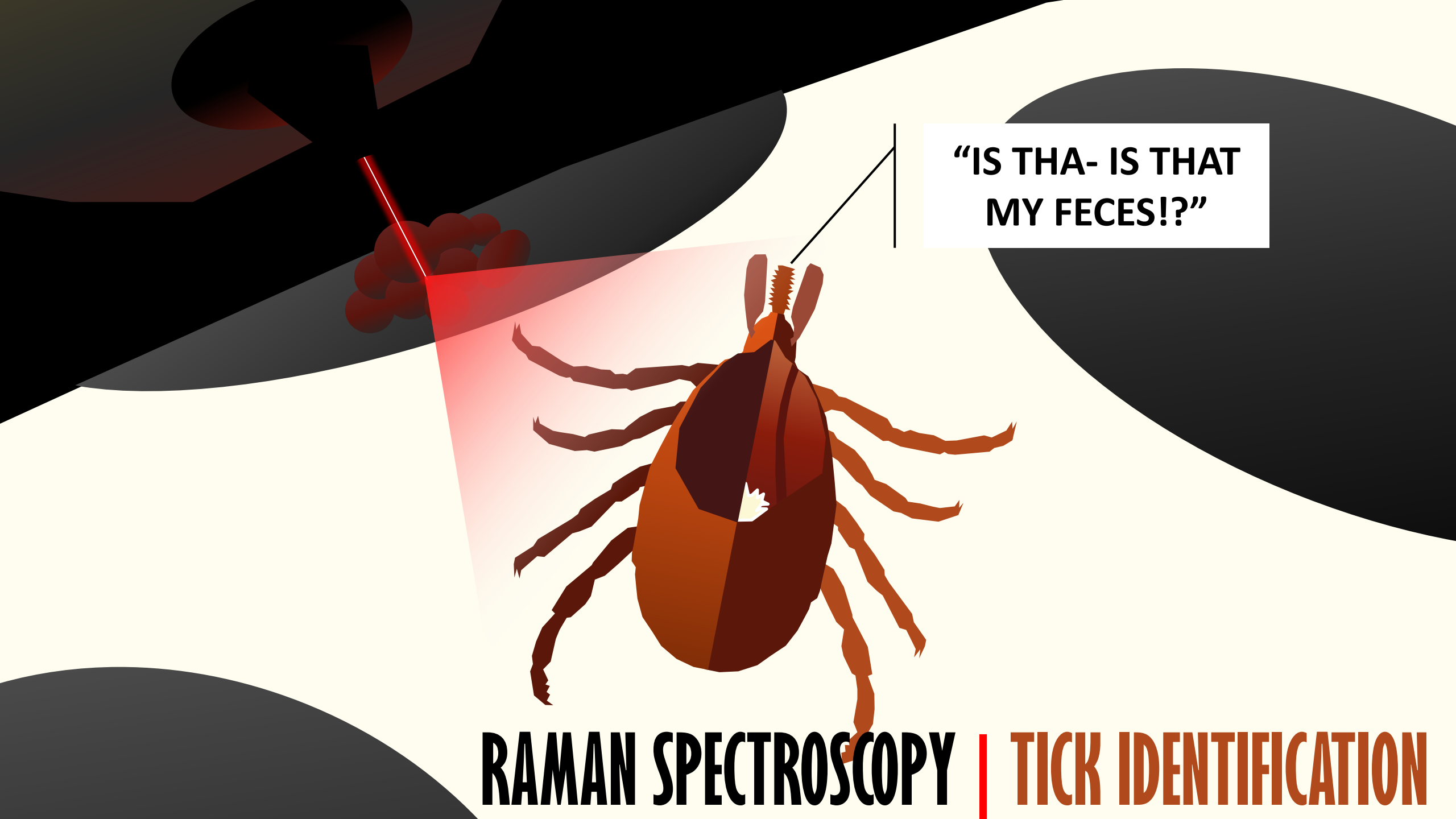
Future Directions

- Use GC-MS to more accurately evaluate the compositions and quantitative comparisons within and across all 12 tick species' frass samples.
- Check for in-species genetic flow variations in Raman spectra
- Expand our life stages from nymph-adult to larvae-adult
- Check for variation between male and female fecal spectra from the same species.

Literature

- **Anderson, D. P., Hagerman, A. D., Teel, P. D., Wagner, G. G., Outlaw, J. L., and Herbst, B. K. (2010).** Economic Impact of Expanded Fever Tick Range. Research Report 10-2. College Station, TX: Agricultural and Food Policy Center, Texas AgriLife Research.
- **Annand, P. N. (1941).** Report of the Chief of the Bureau of Entomology and Plant Quarantine 10 [40-] 41. Report of the Chief of the Bureau of Entomology and Plant Quarantine 10 [40-] 41.
- **Kaufman, W. R., & Phillips, J. E. (1973).** Ion and water balance in the ixodid tick Dermacentor andersoni: I. Routes of ion and water excretion. Journal of Experimental Biology, 58(2), 523-536.
- **Palmer, W. A., Treverrow, N. L., & O'Neill, G. H. (1976).** Factors affecting the detection of infestations of Boophilus microplus in tick control programs. Australian veterinary journal, 52(7), 321-324.
- **Sonenshine, D. E., & Roe, R. M. (2014).** External and internal anatomy of ticks. Biology of ticks, 1, 74-98.

THANK YOU FOR THIS OPPORTUNITY!



RAMAN SPECTROSCOPY | TICK IDENTIFICATION