Immunolocalization of sodium-dependent cation chloride cotransporters in the yellow fever mosquito *Aedes aegypti* larvae

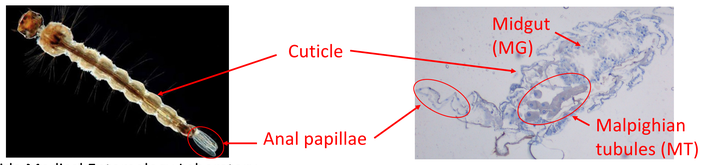
Grace Riley ‘18 and Dr. Chris Gillen
Department of Biology, Kenyon College, Gambier, OH

Abstract

The *Aedes aegpyti* mosquito carries several viruses that cause disease in humans, including Zika, dengue, and yellow fever. Mosquitoes must tightly regulate their salt levels to survive, so salt transport processes are a possible target for novel insecticides. We have identified three sodium-dependent cation-chloride cotransporters that are believed to play an important role in *A. aegypti* osmoregulation. We tested methods of immunohistochemistry to develop a reliable procedure for detecting these ion transporters in larvae. The cotransporter’s location suggests its potential function. Preliminary results indicate a presence of these proteins; however, their specific location is unclear. The use of purified antibodies should greatly clarify the results.

Background

- Osmoregulation may be a target to prevent spread of disease via mosquito. Larvae reared in freshwater—a hypotonic environment—face a constant passive loss of ions, so active ion uptake is vital.
- Malpighian tubules secrete excess fluid to concentrate hemolymph. The hindgut and anal papillae (larvae only) reabsorb necessary water and ions.
- We investigate the role of three sodium-dependent cation chloride cotransporters (*ae*CCC1-3) in mosquito osmoregulation. Phylogenetic analysis indicates *ae*CCC2 and *ae*CCC3 diverged from *ae*CCC1, thus suggesting a greater functional difference between *ae*CCC1 and *ae*CCC2/3. We hypothesize that *ae*CCC3 arose upon the development of the mosquito’s aquatic larval stage.



Florida Medical Entomology Laboratory

Hypotheses and Predictions

Protein	Function	Location	Lifecycle expression
*ae*CCC1	Secretory	Malpighian tubules - apical membrane	Equal expression in all stages
***ae*CCC2**	**Absorptive**	**Hindgut and anal papillae - apical membrane**	**Higher in adults than larvae**
***ae*CCC3**	**Absorptive**	**Hindgut and anal papillae - apical membrane**	**High in larvae Very low in adults**

Methods

Mosquito rearing and dissections
- *Aedes aegypti* (Liverpool strain) eggs hatched under vacuum pressure in freshwater
- Larvae kept at 28°C and 80% humidity on a 12:12 light:dark cycle and fed TetraFin fish flakes every other day
- Second and third instar larvae dissected in cold Ringer solution and tissues incubated in fixative overnight at 4°C

Antibody development
- Antibodies for *ae*CCC2 and *ae*CCC3 made concurrently in the same rabbit
- Experiments used raw serum that contained mixture of antibodies

Tissue preparation and slicing
- Tissues washed in PBS, dehydrated in ethanol, washed in xylene, and embedded in paraffin
- Chilled paraffin blocks sliced (7µm) and placed onto warmed microscope slides coated with albumin
- Slides dried overnight at 56°C

Slide processing and imaging
- Tissues deparaffinized in xylene and rehydrated in ethanol
- The rest of the procedure varies per method of detection

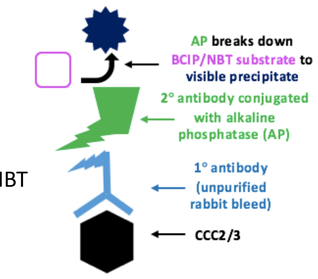
Slides imaged with a Nikon DS Camera Control Unit DS-L2

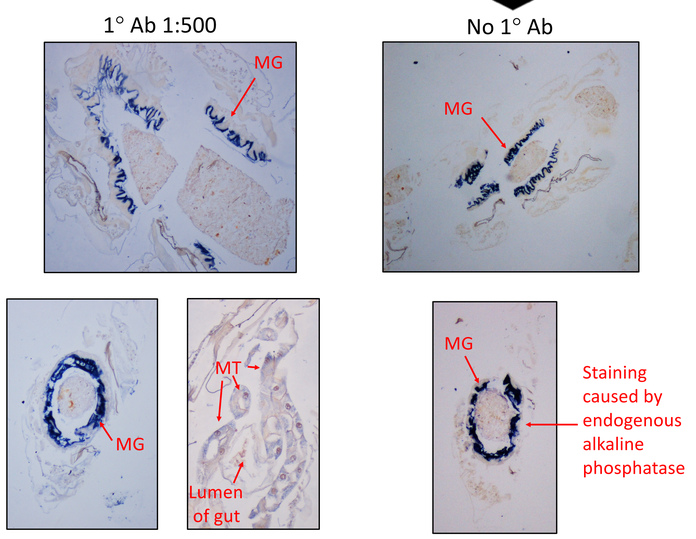
Alkaline Phosphatase

Methods, cont.
- Slides were incubated with:

1. 10% normal goat serum (NGS)
2. 1° Ab *ae*CCC2/3 test bleed in NGS
3. 2° Ab alkaline phosphatase (1:500 in NGS)

- Dipped in AP buffer
- Flooded with chromogenic substrate BCIP/NBT and then dipped in stop solution
- Dehydrated in ethanol and xylene





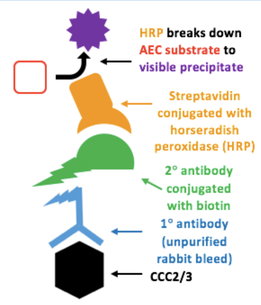
Endogenous alkaline phosphatase metabolized BCIP/NBT giving false positive results. Some differences were detected between experimental and control preps. We could incorporate a step to block endogenous alkaline phosphatase.

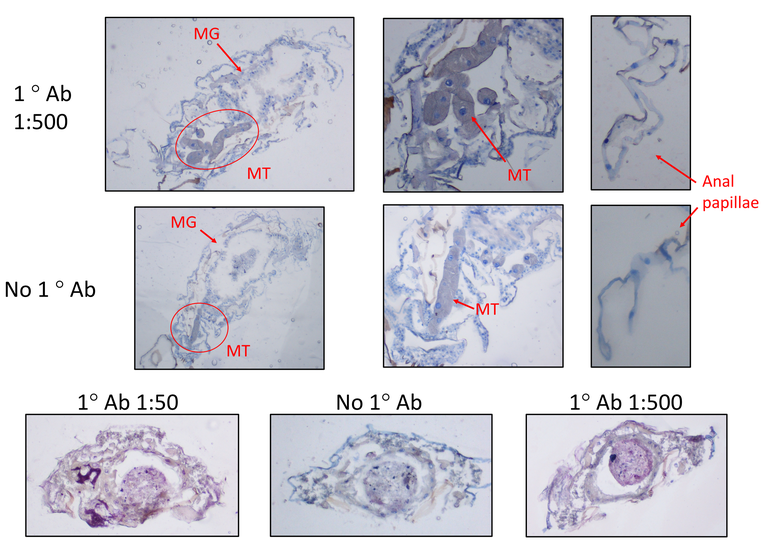
Labeled Streptavidin Biotin with Horseradish Peroxidase

Methods, cont.
- Endogenous peroxidase inhibited with 0.5% H_2O_2 in methanol
- Slides were incubated with:

1. 10% normal goat serum/2x casein in PBS
2. 1° Ab *ae*CCC2/3 test bleed in PBS/1x casein
3. 2° Ab biotinylated goat anti-rabbit IgG
4. Streptavidin/horseradish peroxidase
5. Chromogenic substrate AEC

- Counterstained with hematoxylin



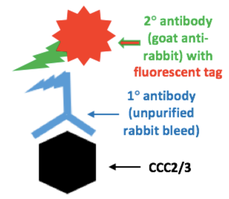


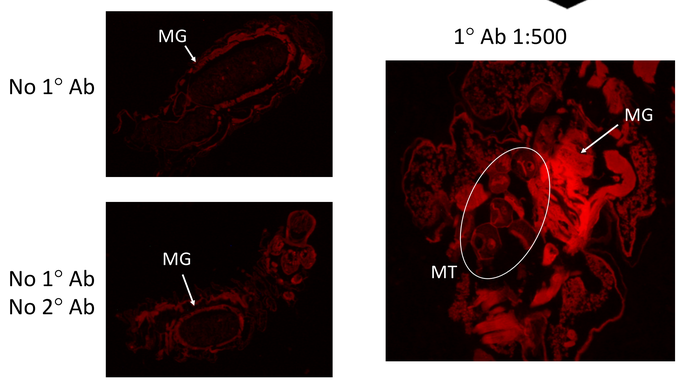
The experimental tissue exhibits a more purple hue than the control. No strong differences were detected between 1° antibody concentrations.

Fluorescence

Methods, cont.
- Slides were incubated with:

1. 10% normal goat serum (NGS)
2. 1° Ab *ae*CCC2/3 test bleed in NGS
3. 2° Ab TRITC (rhodamine) (1:400 in NGS)





The tissue exhibits a high level of autofluorescence, thus making it difficult to differentiate between levels and colors of fluorescence.

Summary and Future Directions

Summary
- Larvae were successfully embedded and sliced, and internal structures can be identified
- General staining differences were observed between experimental and control slides
- Differences are observed between experimental and control slides, but no conclusions can be drawn about the specific location of *ae*CCC2/3

Future Directions
- Repeat experiments with recently purified primary antibodies that will give more specific results
- Develop antibody against *ae*CCC1 for experiments
- Continue Western blot and qPCR experiments to corroborate immunohistochemistry findings

Acknowledgements

Special thanks to Dr. Harry Itagaki for his invaluable help in teaching me the immunohistochemistry procedure. I would like to thank my mentor, Dr. Chris Gillen, for his steady guidance throughout the whole summer. We would like to acknowledge our collaborator, Dr. Peter Piermarini, at the Ohio Agricultural Research and Development Center. Finally, thank you to my fellow mosquito researchers for inspiring me and keeping me company. This research was funded by the National Science Foundation (IOS-1557230) and the Kenyon College Summer Science program.

References

- Beyenbach, K. W. Transport mechanisms of diuresis in Malpighian tubules of insects. *J Exp Biol*. 2003, *206*, 3845-3856.
- Beyenbach, K. W.; Piermarini, P.M. Osmotic and ionic regulation in insects. In *Osmotic and Ionic Regulation: Cells and Animals*. Evans, D. H., Ed.; CRC press, Boca Raton, 2009; pp 231-293.
- Bradley, T. J. Physiology of osmoregulation in mosquitoes. *Annu. Rev. Entomol*. 1987, *32*, 439- 462.
- Musso, D.; Cao-Lormeau, V. M.; Gubler, D. J. Zika virus: following the path of dengue and chikungunya? *The Lancet*, 2015, 386, 243-244.