# Localization of *Aedes aegypti* Na-dependent aeCCC1 by affinity-purified antibodies to Malpighian tubules and hindgut

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## Abstract

*Aedes aegypti* (Yellow Fever Mosquito) is a vector for an array of diseases that claim the lives of millions every year. The eradication of such diseases requires controlling the tenacious animal. However, high public-health costs and increasing resistance of mosquitoes to current pesticides has led researchers to investigate alternatives. Insecticides targeting osmoregulatory proteins responsible for regulating salt and water balance are a promising alternative. Sodium-dependent cation-chloride cotransporters (CCCs), thought to be responsible for the uptake and excretion of salt and water, are the focus of our studies. Sampling tissue from laboratory-bred colonies, we investigated protein expression of aeCCC1 proteins through immunoblotting. Immunoblot tests were conducted with the aid of affinity purified antibodies that were paralogs to aeCCC1. Detection of strong bands above the 250kDa range of hindgut and Malpighian tubules in both adult male and female samples confirmed expected protein weights. Distribution and localization of these antibodies in tissue will be confirmed via immunohistochemistry.

## Background

Illnesses like malaria, dengue, yellow fever, and zika all share *Aedes aegypti* as its vector. (VDCI 2016) Development of an effective insecticide requires the systematic targeting of specific proteins involved in key physiological functions, such as water and ion balance. Osmoregulatory challenges are unique in the adult stages, with blood-feeding females taking in volumes almost 2 times greater than their body weight. (Williams et al. 1993) This calls for the excretion of excess salt and water to maintain homeostasis in the engorged animal. Renal (Malpighian) tubules and the hindgut are promising locations for such activity as their transepithelial membrane proteins are known to carry $Na^+$ cations. (Wiliams et al. 1993) In vertebrate systems, a cotransporter responsible for secretion and absorption of ions across membranes is the Na-K-Cl cotransporter (NKCC). NKCC isoforms belong to a superfamily of highly conserved ion-transporting proteins called cation-chloride cotransporters. Mounting evidence shows that these same proteins are synthesized from similar genes to a subfamily of proteins found in *Aedes aegypti*. (Hartman et al. 2014) Designated Na+-dependent cation-Cl- cotransporters (aeCCCs), past research has consistently shown that these proteins also participate in epithelial ion transport. (Beyenbach and Piermarini 2011) Data suggests that distal portions of Malpighian tubules secrete fluid from hemolymph to gut lumen, while the proximal tissue and hindgut participate in absorption of salt into the hemolymph. (Gillen et al 2017) We evaluated protein expression using polyclonal antibodies to the putative aeCCC1 gene in adult tissues. *Manduca sexta* antibodies M6 and A5 used to further detect bands at the 250 kD level.

## Methods

**Colony :** *Aedes aegypti* were raised in a laboratory setting as per previous literature (Pannabecker et al. 1993). CDC eggs where hatched using a vacuum chamber to induce a hypoxic state. Larvae were raised in tap water to adult stage in 28°C and 80% humidity in a 14:10 H light/dark cycle. Larvae were fed *ad libitum* with finely ground fish flakes and yeast extract. Pupae were transferred to a collapsible mesh cage (Bug Dorm) for eclosion. Adults were fed 10% sucrose/tap water in an inverted test tube with a cotton stopper.

**Tissue Isolation and Sample Preparation:** Mosquito sacrificing and dissection procedure followed according to earlier works (Piermarini et al. 2009). Adults were cold-anesthetize before being submerged in Ringer solution containing 150 NaCl, 3.4 KCl, 1.7 $CaCl_2$, 1.8 $NaHCO_3$, 1.0 $MgSO_4$, 5 glucose and 25 HEPES (pH 7.1) in mM. The digestive tract was isolated by pulling on the rectal segment, the five Malpighian tubules were removed with fine forceps, as was the hindgut, with gentle separation from the midgut and segment cuticle. Tissues of 40-50 adults were separated by type and sex and transferred to 1.5-ml microcentrifuge tubes containing 0.5-ml Ringer solution with 20% Protease inhibitor. Samples were homogenized and filled at a 1:1 ratio of 2x Laemmli sample buffer with 5% β-mercaptoethanol.

**Western Blotting:** Procedure was performed as described by Perimarini at al. (2011), whereby adult male and adult male tissue lysates were loaded into 7.5%-polyacrylamide 12-well gels. Proteins were then transferred to an immunoblot PVDF membrane. Membranes were washed in phosphatase buffered saline-Tween (PBS-t) and blocked in bovine serum albumin (BSA) for 1 H. Membranes were incubated with affinity-purified primary antibodies aeCCC1-1, aeCCC1-2, A5 at concentrations of 1:500 and M6 at 1:1000 overnight at 4°C with gentle agitation. Blots were incubated in anti-mouse HRP and HRP anti-rabbit IgG secondary antibody at 1:1000 concentrations. Bands were detected using chemiluminescence using ChemiDoc MP imager (Bio-Rad) white light conversion screen.

**Densitometric Analysis:** Chemiluminescent blots were washed with PBS and incubated in 10% methanol 7% acetic acid for 15 minutes under gentle agitation. Four 5 minute washes of DI $H_2O$ were performed followed by a 15 minute incubation in SYPRO Ruby Protein Blot stain on a rocker. Blots were washed three times for 5 minutes of DI $H_2O$. Imaged with the ChemiDoc MP imager on XcitaBlue Conversion screen. ImageLab used to quantify protein volumes.

**Antibody Production:**

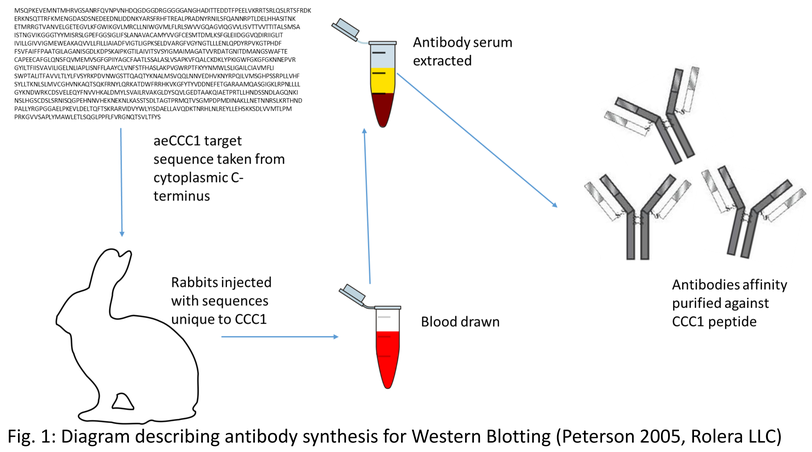


Fig. 1: Diagram describing antibody synthesis for Western Blotting (Peterson 2005, Rolera LLC)

## Results

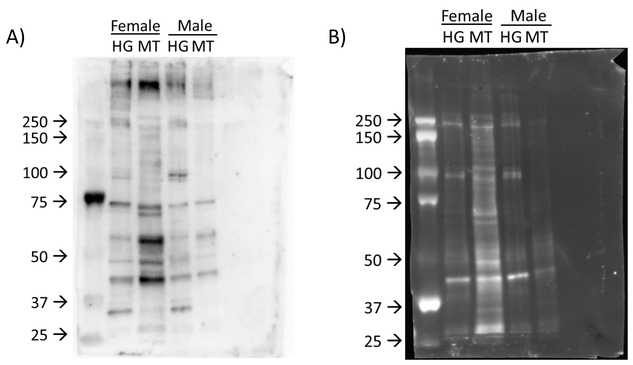


Fig 2: A) Western blot protein expression of aeCCC1 in adult female and male *Aedes aegypti*. Cell lysates from Malpighian tubules (MT) and hindgut tissue (HG). Using the aeCC1-1 antibody to display bands, those above the 250 kD range indicate aeCCC1 expression in respective tissues. aeCCC1 intensity highest in adult female Malpighian tubules, indicating high protein expression in this tissue. 24.669 second exposure. B) Corresponding SYPRO bands intensities express relative protein concentration per well.

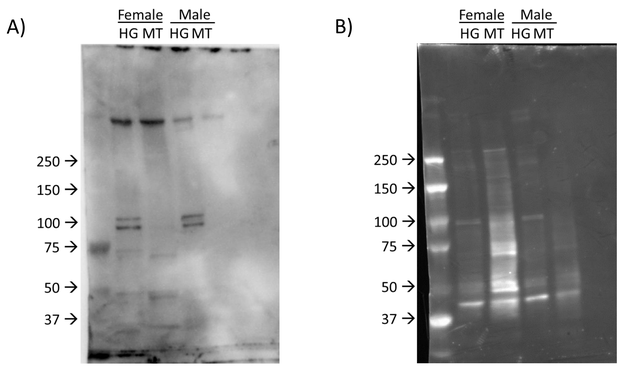


Fig. 3: A) Western blot of female and male MT and HG lysates. Blot was immunostained with aeCC1-2, recognizing aeCCC1 in adult *Aedes aegypti* tissues. Those above the 250 kD range indicate aeCCC1 expression in respective samples. aeCCC1 intensity highest in adult female MT, suggesting high protein expression in this tissue, evidence for HG as well. Bands weakly expressed in males, particularly in HG. 4.632 second exposure. B) SYPRO blot indicating relative protein quantities in well-loads.

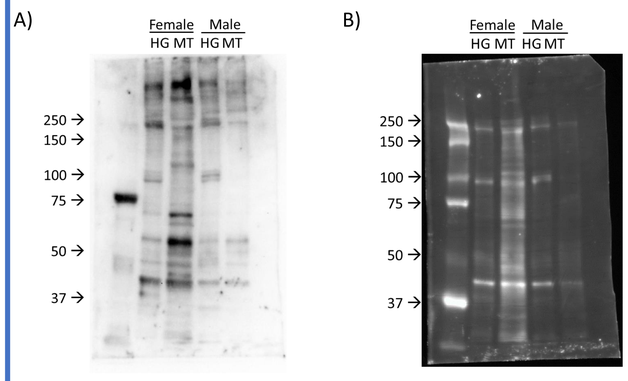


Fig. 4: A) Western of female and male HG and MT. Blot was immunostained with M6, an antibody against *Manduca sexta*. Figure displays aeCCC1 expression in adult *A. aegypti* tissues in both HG and MT. Those above the 250 kD range indicate aeCCC1 expression in respective tissues. CCC1 intensity highest in adult female MT, indicating high protein expression in this tissue. Bands present but less distinct in adult female HG. Exposed for 65.528 sec. B) SYPRO measuring relative well protein density.

## Acknowledgments

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## Results

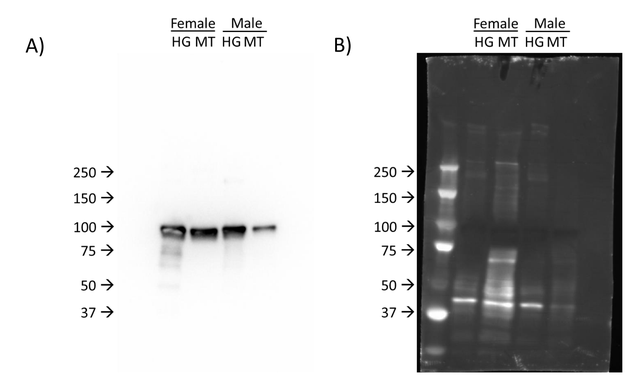


Fig. 5: A) Western blot of MT and HG tissues. Blots were immunostained with A5, and exposed for 0.594 sec. Displays aeCCC1 expression in adult *A. aegypti* tissues. Using the A5 antibody to display bands, low exposure times gave us these bands far below the 250 kD range, serving as a positive control for the blot. SYPRO band intensities indicaes. B) SYPRO displays relative protein content per well.

## Conclusion

- Data suggest that *Aedes aegypti* protein aeCCC1 is present at molecular weights greater than 250 kDa.
- Affinity purified antibody aeCCC1-1 detects the presence of a single prominent band in the above 250 kDa region in adult female Malpighian tubules.
- aeCCC1 is essentially absent in male Malpighian tubules, perhaps owing to the their central physiological function as salt and water balance after blood meals in females (Piermarini and Gillen 2015)
- aeCC1-1 and aeCC1-2 immunoblots suggest expression of aeCCC1-1 in both HG and MT in females. Strongest expression appears to be in MT
- aeCCC1 is displays strongest expression in adult female Malpighian tubules
- SYPRO Ruby staining shows an imbalance of proteins per well, calling for standardized blots to be used in immunostaining.

## Future Directions

- Investigate MT and HG with polyclonal antibodies against aeCCC2 and 3 and determine protein expression.
- Understand localization of antibodies in MT and HG tissues using immunohistochemistry.
- Continue densitometric analyses to quantitatively detect protein per well.
- Standardized volumes would serve to control for protein level irregularities.
- Peptide competition dot blots.

## References

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