Molecular characterization of Na^+-coupled CCCs in the yellow fever mosquito *Aedes aegypti*

Daniel C. Akuma ’14, Christopher M. Gillen
Kenyon Summer Science 2013

Abstract

Aedes aegypti transmits two of the most important mosquito-borne viral diseases in the world, namely yellow fever and dengue. In order to survive, reproduce and carry disease, they require sophisticated osmoregulatory mechanisms. Cation chloride cotransporters (CCCs) play a key role in vertebrate osmoregulation, but are poorly understood in insects. Using qPCR, we evaluated expression levels of three genes encoding putative Na^+-coupled CCCs, namely: aeCCC1, aeCCC2, and aeCCC3. In adult female mosquitoes, aeCCC1 was expressed in the head region 3 to 20 times greater than in Malpighian tubules, hindgut and midgut regions. On the contrary, aeCCC2 showed 200-fold higher expression in the hindgut than in other regions. In mosquito larvae, aeCCC1 and aeCCC2 were more highly expressed in the Malpighian tubules compared to anal papillae. But anal papillae expressed aeCCC3 about 6,000 times higher than Malpighian tubules. Expression of aeCCC3 in larval Malpighian tubules increased 90-fold in larvae raised in 30% sea water, with no changes in aeCCC1 and aeCCC2. Overall, across stages of development, aeCCC3 was expressed 500 times higher in larvae than in pupae or adults; aeCCC2 was expressed 35-fold higher in larvae and adults than in pupae, whereas aeCCC1 was expressed in similar amounts across all stages. The different gene expression patterns of aeCCC1, aeCCC2, and aeCCC3 in the above conditions suggest diverse functional roles for these transporters.

Introduction

Background

- The yellow fever mosquito, *Aedes aegypti* (Linnaeus), transmits two of the most important mosquito-borne viral diseases according to WHO annual report (2012).
- *A. aegypti* larvae live in freshwater and therefore encounter different salt and water balance challenges than terrestrial adults.
- Blood-sucking female adults face the greatest salt and water balance challenges. In one blood meal, they ingest a blood volume 2 to 3 times greater than their body size. Female mosquitoes must excrete the excess salt and water, both to maintain homeostasis and easily escape predators.
- Mosquitoes osmoregulate by transporting salt across the epithelia of the alimentary canal, especially the midgut, hindgut and Malpighian tubules.
- The distal portion of Malpighian tubules secretes fluid from hemolymph to gut lumen, whereas the proximal tubule and the hindgut predominantly absorb salt and water into the hemolymph.
- Na-dependent cation chloride cotransporters (CCC) play key roles in epithelial salt transport. But, compared to vertebrates, CCCs are poorly understood in insects. Thus, we evaluated 3 genes that encode CCCs in *A. aegypti*, namely: aeCCC1, aeCCC2 & aeCCC3.
- Phylogenetic analysis reveals close homology between aeCCC proteins, other insect CCCs, and vertebrate bumetanide-sensitive Na-K-2Cl cotransporters (NKCC) (Fig. 1). We quantified gene expression levels using quantitative real-time PCR.

Hypotheses

- aeCCC isoforms are differentially expressed across tissues along the alimentary tract of *A. aegypti.*
- The expression levels of aeCCC isoforms vary at different stages of development (i.e. larvae, pupae and adults) due to osmoregulatory function of each isoform (secretory or absorptive).
- Expression levels of aeCCC isoforms in larvae depend on salinity of surrounding water.

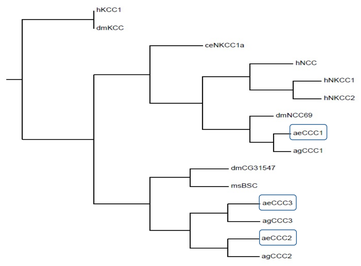

Fig. 1: Phylogenetic analysis of Na-dependent cation-chloride cotransporter (CCC) amino acid sequences by the unweighted pair group method with arithmetic mean. Gaps are excluded from the analysis. Prefixes indicate species: *Drosophila melanogaster* (dm), *Aedes aegypti* (ae), *Anopheles gambiae* (ag), *Homo sapiens* (h), *Caenorhabditis elegans* (ce), *Manduca sexta* (ms). Modified from Gillen and Piermarini NSF Proposal.

Methods

Insects: Wild-type *Aedes aegypti* were raised from egg to adult stage as described (Pannabecker *et al.*, 1993). Eggs (Puntarenas, Costa Rica) were hatched under a vacuum chamber to induce low O_2. Larvae were raised in ~$1^1/_2$ inch freshwater at 28 °C, 80% RH and 12hr light/day diurnal cycle. Larvae were fed *ad libitum* with finely ground Total Goldfish® (Secaucus, New Jersey) and water was changed once every 5 days or when too cloudy. After 6-7 days, pupae were transferred into a collapsible mosquito cage (Bioquip Prod., California) for eclosion. Adult mosquitoes were fed 10% sucrose (w/v). For salinity experiments, larvae were raised in two identical containers containing freshwater and 30% sea water respectively. Each colony was maintained in the growth conditions described above until 4th instar.

Dissection: Samples from the head and key osmoregulatory tissues (Malpighian Tubules, midgut and hindgut) were obtained from adult females (3 – 9 days old) as previously described (Piermarini *et al.*, 2013). Each tissue was collected in 1-mL cold TRIzol reagent (Invitrogen, Carlsbad, CA) for RNA isolation. Fourth instar larvae were dissected using a slight modification of the protocol described by Del Duca *et al.* (2011). The anal papillae and Malpighian tubules were isolated and collected in TRIzol reagent (1 mL) for RNA isolation.

RNA Isolation and qPCR: Tissue-specific or total *Aedes* RNA was isolated using the TRIzol prep according to manufacturer’s instructions. RNA was quantified via NanoDrop-1000 (Thermo Scientific, Wilmington, DE) and decontaminated of genomic DNA using the TURBO DNA-free kit (Ambion/Applied Biosystems, Austin, TX). RNA was further purified using the RNA Clean & Concentrator™-25 kit (Zymo Research Corp., Irvine, CA). Total RNA (10 µg) or tissue-specific RNA (1 µg) was reverse-transcribed using the TaqMan Reverse Transcription kit (Applied Biosystems, Life Technologies, Carlsbad, CA). Primers were designed using the Primer Express Software v2.0 (Invitrogen). For all qPCR experiments, expression levels were quantified using the fluorescent marker SYBR Green (Applied Biosystems, Austin, TX) and the ribosomal protein S5 (aeRpS5) was used as internal control (Ribeiro *et al.*, 2007). Q-PCR was run in triplicate on a 96-well plate. Gene expression levels were quantified by threshold cycle differences (dCT) on an ABI prism 7500 sequence detector.

Results

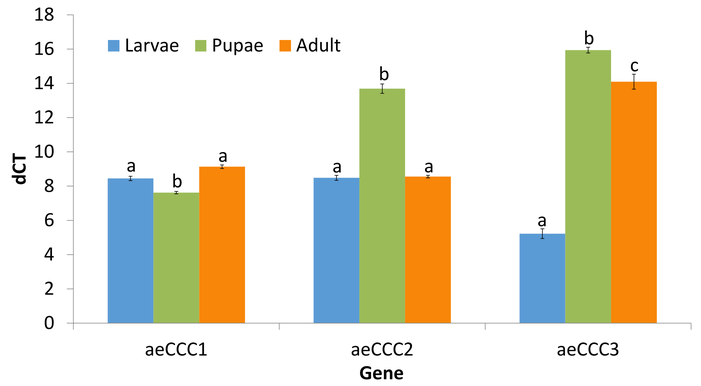

Fig. 2: Differential expression of three isoforms of a putative Na^+-coupled cation chloride cotransporter (CCC) gene across developmental stages in *A. aegypti*. Smaller dCT values indicate higher expression. N (larvae, pupae) = 4. N (adult female) = 3. Each sample was pooled from 10 (larvae, pupae) or 8 (adult female) individual organisms. Ribosomal RNA ‘Rps5’ = internal control. Error bars = SEM. For aeCCC1: ANOVA, F = 17.13, $df_{dev.}$ = 2, df_{error} = 9, p = 0.001. For aeCCC2: ANOVA, F = 118.84, $df_{dev.}$ = 2, df_{error} = 9, p < 0.001. For aeCCC3: ANOVA, F = 260.48, $df_{dev.}$ = 2, df_{error} = 8, p < 0.001. Within a gene, means that do not share a letter are significantly different.

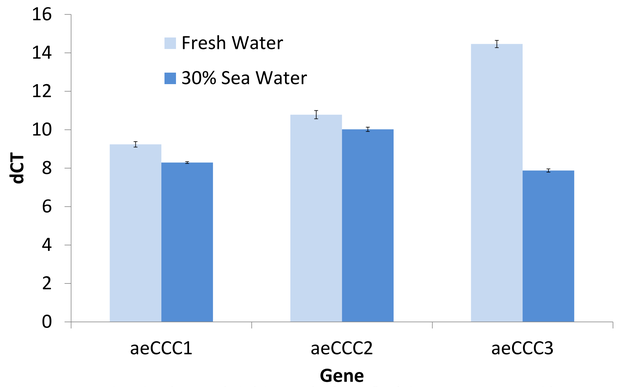

Fig. 3: CCC expression in the Malpighian tubules of 4th instar *A. aegypti* larvae raised in different salinities. Smaller dCT values indicate higher expression. Ribosomal RNA ‘Rps5’ = internal control. N = 3 for each treatment. Each sample was pooled from 30 to 35 larvae. Error bars = SEM. For aeCCC1: Two-sample t test, t = 6.25, df = 2, p = 0.025. For aeCCC3: Two-sample t test, t = 31.78, df = 2, p = 0.001.

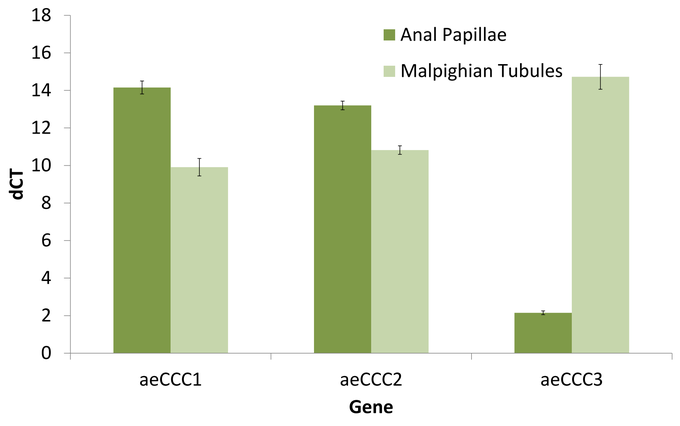

Fig. 4: Comparative expression of putative Na-coupled cation chloride cotransporters (CCCs) in the anal papillae and Malpighian tubules of 4th instar *A. aegypti* larvae. Smaller dCT values indicate higher expression. N = 3 for each treatment. Each sample was pooled from 30-35 larvae. Ribosomal RNA ‘Rps5’ = internal control. Error bars = SEM. For aeCCC1: Two-sample t test, t = 7.30, df = 3, p = 0.005. For aeCCC2: Two-sample t test, t = 7.28, df = 3, p = 0.005. For aeCCC3: Two-sample t test, t = -18.83, p = 0.003.

References

- Del Duca, O., A. Nasirian, V. Galperin & A. Donini. 2011. Pharmacological characterization of apical Na^+ and Cl^- transport mechanisms of the anal papillae in the larval mosquito *Aedes aegypti*. **J Exp Biol** 214:3992-9
- Pannabecker, T. L., T. K. Hayes & K. W. Beyenbach. 1993. Regulation of epithelial shunt conductance by the peptide leucokinin. **J Membr Biol** 132(1):63-76
- Piermarini, P. M., M. F. Rouhier, M. Schepel, C. Kosse & K. W. Beyenbach. 2013. Cloning and functional characterization of inward-rectifying potassium (Kir) channels from Malpighian tubules of the mosquito *Aedes aegypti*. **Insect Biochem Mol Biol** 43(1):75-90
- Ribeiro, J. M. C., B. Arcà, F. Lombardo, E. Calvo, V. M. Phan, P. K. Chandra & S. K. Wikel. 2007. An annotated catalogue of salivary gland transcripts in the adult female mosquito, *Aedes aegypti*. **BMC Genomics** 8:6
- World Health Organization. 2012. *Global Strategy for Dengue Prevention and Control,* 2012 – 2020. WHO Press, Geneva, Switzerland

Results

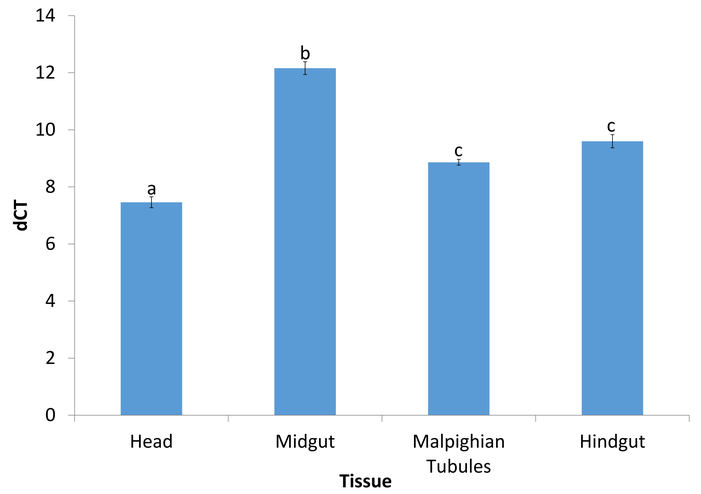

Fig. 5: Tissue distribution of CCC1 gene in adult female *A. aegypti.* Smaller dCT values indicate higher expression. N = 3 independent samples for each tissue type. Ribosomal RNA ‘Rps5’ was used as internal control for qPCR experiments. Error bars = SEM. ANOVA, F = 59.98, df_{tissue} = 3, df_{error} = 8, p < 0.001. Means that do not share a letter are significantly different.

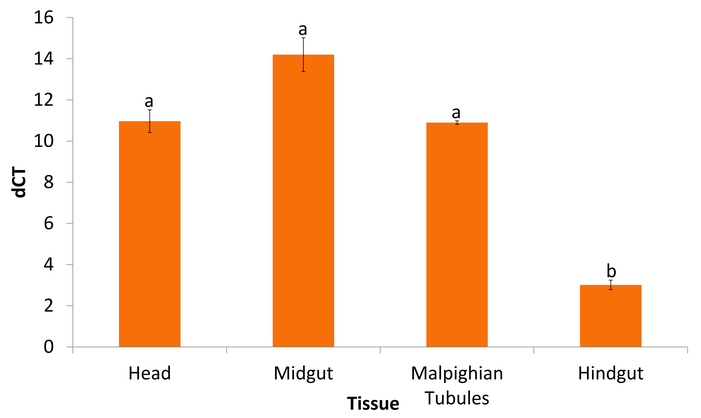

Fig. 6: Tissue distribution of CCC2 in adult female *A. aegypti.* Smaller dCT values indicate higher expression. N = 3 independent samples for each tissue type. Ribosomal RNA ‘Rps5’ was used as internal control for qPCR experiments. Error bars = SEM. ANOVA, F = 27.13, df_{tissue} = 3, df_{error} = 8, p < 0.001. Means that do not share a letter are significantly different.

Summary

- **aeCCC1** shares closer homology to vertebrate NKCCs than other aeCCCs (Fig. 1). aeCCC1 is constitutively expressed across developmental stages (Fig. 2) and more highly expressed in the adult head than any osmoregulatory tissue studied (Fig. 5). Its expression is not influenced by 30% sea water growth conditions.
- **aeCCC2** is downregulated during the larva-to-pupae metamorphosis but upregulated again in adults (Fig. 2). In adults, aeCCC2 is expressed more than 200-fold higher in the hindgut than in other osmoregulatory tissues (Fig. 6). But in larvae, aeCCC2 expression is not affected by 30% sea water growth conditions (Fig. 3).
- **aeCCC3** is more highly expressed in larvae than in pupae and adult females (Fig. 2). In larvae, aeCCC3 expression is higher in the anal papillae than in Malpighian tubules (Fig. 4). However, this tubular expression is upregulated in 30% sea water growth conditions (Fig. 3).

Conclusion/Future Questions

- The different tissue and developmental expression patterns of aeCCC1, aeCCC2 and aeCCC3 suggest diverse functional roles for these transporters.
- These preliminary results necessitate immunohistochemical studies with isoform-specific antibodies to localize the aeCCC proteins.
- The current study has not shown the immediate effect of a blood meal on aeCCC expression levels. In the future, it will be necessary to determine the time-dependent expression levels of aeCCC isoforms along the female alimentary canal after a blood meal.

Acknowledgments

I would like to thank Dr. Chris Gillen, my mentor whose professional advice brought this work to reality. My regards also go to Dr. Peter Piermarini, Dr. Matt Rouhier and other members of the Piermarini Lab, Ohio Agricultural Research Development Center, Wooster, OH for guidance in starting a mosquito colony. Lastly, I thank Andrew Parmelee ’14 and Jacque Garcia ’15 for good company this summer. This project was funded by the Kenyon Summer Science Grant (2013).