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Research Article

Every Buggy Pees: Changing Morphometrics of Osmoregulatory Organs in Aquatic and Terrestrial Insects and Changes in Ion Transport Properties in Response to Environmental Changes and Pharmacological Inhibition of Ion Transport

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Abstract: Terrestrial and aquatic insects such as crickets, caterpillars, and mosquito larva utilize specialized osmoregulatory organs known as Malpighian tubules to regulate water and ion levels in their hemolymph, a parameter crucial for proper digestion, absorption, and excretion. Structures such as anal papillae are present in aquatic insects to additionally absorb ions from the environment and excrete waste. To examine the effects of dietary ion content on insect osmoregulation, caterpillars were raised on control, high-water and high-K^+ diets for several experiments. Changes in dietary ion availability did not increase hemolymph osmolality and did not perturb the transepithelial potential Vte or basolateral membrane potential Vbl in the Malpighian tubules. Interestingly, the number of secondary cells present in the MTs of caterpillars raised on a high-water diet was significantly lower. Malpighian tubule function was additionally examined in crickets using simple dye accumulation assay. Inhibition of active ion transport with DNP, as well as treatment with ouabain (a specific Na^+/K^+-ATPase inhibitor) and incubation with Na^+-free saline decreased the MT function. In mosquito larva, anal papillae serve as both excretory and osmoregulatory organs. To examine whether the size of the anal papillae would change under increasing external ion concentrations, we measured the length and the width of this organ to find that under increasing environmental salinity, there was no significant change in the width of the anal papillae however there was a decrease in length. Although they do not diminish completely because the anal papillae have other functions in mosquito larva in which they play a role in excretion and respiration. These results proposed that the insects have osmoregulatory organs that can change in size in response to environmental and dietary ion availability.

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Insects constitute the most diverse group of organisms on the planet playing pivotal roles in ecosystems, agriculture, and human health (Scudder, 2017). In insects, excretion of metabolic wastes relies on multiple specialized organs like Malpighian tubules, anal papillae, hindgut, which employ transporters like Na^+/K^+-ATPase, and aquaporins to transport ions and water across their epithelia (Weihrauch & O'Donnell, 2021). All these structures work together to direct the flow of ions and water to ensure that metabolic wastes are excreted properly while ion and water homeostasis is maintained (Cohen et al., 2020). While many insects have beneficial ecological roles, many are considered pests and insecticides are developed and introduced into the environment to regulate them. Through a variety of transfer pathways, pesticides can move to other environments and

off-target species (Hashimi et al., 2020). Investigating insect excretion allows scientists to further understand the difference between insect clades and how to avoid off-target effects when developing effective integrated pest management strategies.

The Malpighian tubules (MTs) serve as a kidney-like excretory organ in insects. The tubules allow for osmoregulation, excretion of waste, reabsorption of nutrients, and even hormone production. The main functions of MTs as excretory organs are conserved in terrestrial and aquatic insects (Klowden, 2013). Waste excretion requires water, which is passively transported into the MTs via aquaporins. The water is transported following the active transport of ions from the hemolymph into the lumen of MTs using ion pumps channels and transporters - this creates an ion gradient necessary for water to follow osmotically into the lumen. MTs consist of an epithelium layer that takes part in the regulation of ion and water balance within the insect's body. In caterpillars, MT epithelium is made up of principal and secondary cells. Principal cells secrete cations such as Na^+ and K^+ creating an osmotic gradient that drives the transport of water (Kolosov et al., 2018). Secondary cells reabsorb cations and secrete Cl^- in exchange for HCO^- across the tubule epithelium establishing an osmotic gradient for partial reabsorption of water (Kolosov et al., 2018a; Kolosov and O'Donnell, 2020). Net transport of ions creates a membrane potential across MT epithelia enabling the tubules to selectively reabsorb ions and water while excreting waste products.

Anal papillae are organs at the posterior end of aquatic mosquito larvae that take up ions from surrounding dilute aquatic environments (Durant et al., 2021). They play important roles in osmoregulation and ammonia excretion in aquatic insects that have a higher concentration of ammonia in their waste. This means that anal papillae are sites of ammonia detoxification. Studies have shown that changes in gene expression and ion transport in anal papillae can occur when salinity levels increase. The reduced surface area of the papillae reduces the amount of ions taken up from the environment. However, anal papillae also serve a role in excretion so they cannot be removed, suggesting that the size reduction may be limited in high salinity aquatic environments.

Caterpillars (*Trichoplusia ni*) do not have a specific diet and will have a diverse palate to maintain homeostasis and store energy for their development into butterflies. To do this, caterpillars adjust their water content by controlling how much water they lose to their feces (Reynolds & Bellward, 1989). To maintain homeostasis, they must regulate their osmolality which involves the MTs secreting and reabsorbing ions and water between their hemolymph and MT fluid. The hemolymph will help transport nutrients, hormones, and waste products through the caterpillar's body. To maintain osmolality, the hindgut, midgut, and rectal complex are also utilized for ion and water transport. The midgut maintains a basic pH which allows the caterpillar to digest plant-based foods better. The MTs form a complex with the rectum (rectal complex) which facilitates reabsorption of water from excretions to conserve water (Ramsay, 1976). The rectal complex is the collection of distal ends of MTs and the rectum that regulate the water balance within the caterpillar's body by absorbing water and ions from the digested food material before it is produced as frass (Kolosov & O'Donnell, 2019). The caterpillar MTs serve as a very complex kidney system compared to other insects. The MTs are connected to the rectum via rectal complex, which enables them to extract water and ions from the diet before excreting it out of their body. When there is not enough water or ions in the gut to be able to use for fluid secretion and excretion in the MTs, caterpillars switch to sourcing water from their hemolymph. This enables the MTs to use water and ions from their blood to effectively excrete wastes. Previous research showed that specifically the principal cells of MTs in caterpillars, can switch between ion reabsorption and ion secretion, using gut or hemolymph, respectively, as a source of ions for fluid secretion allowing for caterpillars

to condition to any changes within their dietary ion availability (Kolosov et al., 2021). The switch between ion secretion and ion reabsorption involves changes in active transport, water permeability, and septate junction (Kolosov et al., 2021). This can provide an understanding and further research on how caterpillars' internal homeostasis functions to acclimate to changes in diet.

Crickets, being terrestrial omnivores, consume a diet that consists of leaves, seeds, flowers, and insect larvae. The cricket's specific physiology and osmoregulatory organs are what impact their salt and water balance. They use a combination of MTs and their hindgut, which gives them their own version of a kidney. MTs are responsible for digestion, absorption, and excretion which, in an open circulatory system, uses a transport system for the movement of ions up and down a concentration gradient. For instance, crickets are found to secrete high rates of magnesium, using distal segments of their tubules, to maintain homeostasis and eliminate excess magnesium present in their diet (O'Donnell, 2008). Most insects can regulate specific levels of ions through their excretory organs, which allows for osmoregulation and selective reabsorption.

Mosquito larvae *(Aedes aegypti)* are aquatic that reside in a wide variety of water environments, including freshwater and brackish water, and are distributed across 19 California counties (Farrell et al, 2024). They regulate their hemolymph osmolarity within a range of 250 to 300 mOsm using osmoregulatory organs including anal papillae and MTs. Anal papillae facilitate active ion uptake in dilute environments like freshwater using ion transporters adjust ion transport according to the needs of the larvae and depending on the habitat. MTs and anal papillae play a vital role in regulating ion and water levels by actively transporting salts from the hemolymph, creating an osmotic gradient that drives water movement. This process ensures proper nutrient balance in larval development (Misyura et al., 2020).

We used caterpillars, crickets, and mosquito larvae to examine the function of osmoregulatory and excretory organs. In caterpillars, the serum osmolality and number of secondary cells were analyzed to observe differences based on varying availability of ions and H2O present in their diet. It was hypothesized that there would be an increase in serum osmolality in the caterpillars raised on high-K^+ diet due to the influx of ions present in the diet into their hemolymph. Additionally, the number of secondary cells were observed on the caterpillar MTs to determine if diets with high H2O or high ion content would change the number of these reabsorptive cells in the MTs of caterpillars. Previous studies reported that when caterpillars are raised on high-K^+ diet demonstrate increased K^+ reabsorption (primarily found in secondary cells), while much less K^+ is reabsorbed by secondary cells of the MTs in low-ion diet (Kolosov and O'Donnell, 2018). We hypothesized that there would be fewer reabsorptive secondary cells in the MTs of caterpillars raised on high H2O (~low ion) diet and a more secondary cells on the high K^+ diet for increased reabsorption of ions. Additionally, in the MTs of caterpillars transepithelial and basolateral epithelial membrane potential was measured to investigate if diets of high water would alter the movement of ions across the membrane. We hypothesized that in diets with high H20 there would be a higher transepithelial potential because less ions present in the diet therefore typically corresponds with higher ion secretion rates in the MTs (Kolosov et al, 2018). In order to investigate which types of transport and which transporters are involved in the generation of fluid secreted by the MTs, we used crickets, where the MTs were exposed to pharmacological inhibitors and solutions lacking essential ions, and we hypothesized that DNP would stop active transport altogether since it inhibits ATP production in mitochondria; however, if we supplemented the prep with ATP along with the DNP, this would act as a rescue and restart active transport. We hypothesized that ouabain (a specific inhibitor of Na^+/K^+-

ATPase) would block most of the Na^+ and K^+ transport; and likewise, the addition of Na^+-free treatment would prevent Na^+ channels from working in the MTs. In mosquito larvae, we measured anal papillae size in environments of differing salinities to examine whether environmental ion levels would alter the size of the structure. We hypothesized that the anal papillae would be the largest in anal papillae need to be larger in a low salinity environment for greater ion uptake.

Methods

Caterpillars: Serum Osmolality

The goal was to measure the number of dissolved solutes in the blood of caterpillars (*Trichoplusia ni*). First, caterpillars were raised under two different dietary conditions, control (n=8) and high water (n=8). Then, each group of students obtained at least two caterpillars from each dietary condition. The caterpillars were submerged into a dissection dish filled with hydrated mineral oil until they were anesthetized and stopped moving. Then, the caterpillars were pinned to the dissection dish, one pin behind its head and the other on its hind leg. Two sets of forceps were used to rip open the exoskeleton, near the head, and the caterpillar was gently massaged by forceps for the hemolymph to flow out of the tear. A P-1000 pipette was used to collect the hemolymph and put it into labeled 1.5 mL Eppendorf tubules. Then, an additional posterior tear was made in the exoskeleton and the process was repeated until more hemolymph was collected. The hemolymph was then deposited into the same tube. This process was repeated for individual caterpillars in both treatment groups. Then, after all the hemolymph that was collected, the samples were spun at 10,000g for 10 minutes in a centrifuge to remove blood cells and debris from hemolymph. The supernatant was then removed from each tubule. Finally, the samples were loaded into a calibrated osmometer to determine the hemolymph serum osmolality. This reflects the ion and water balance of the insect and is indicative of all ions, nutrients, and wastes dissolved in the hemolymph. Data analysis was performed and average values for treatment groups and standard error of the mean (s.e.m) were plotted in software apps Excel and JASP was used to run statical analysis consisting of a one-way ANOVA coupled with multiple comparison Tukey post-hoc tests.

Caterpillar MTs Morphometrics: Reabsorptive Secondary Cell Count

Larval *Trichoplusia ni* were provided for the dissection of the MTs (n=28 in total). Larvae were placed into a dish filled with saline to initiate dissection for each dietary condition: control, high- ion, and high-water. Under a microscope, microdissection pins were used to pin down the head and one of pseudo-legs to fasten and stabilize the organism in the dish. Iris micro-scissors were then used to make longitudinal incision along the dorsal side of the caterpillar, to cut through the exoskeleton and expose all sections of the gut and the MTs. The exoskeleton was pulled apart to disconnect it from the respiratory tracheae exposing the midgut and hindgut. Once the hindgut was fully exposed, the exoskeleton was fully cut away from the organism. The MTs were then gently separated from the hindgut with a glass probe and all respiratory tracheae were removed with soft tissue forceps. The fully disconnected the ileac plexus regions of the MTs was separated from yellow and white regions, and the posterior end of the tubule was cut at the base of the rectal complex to retain the portion of the MTs containing secondary cells. The dish with the MTs was placed then placed into a black tray to increase the visibility of the secondary cells. The number of secondary cells for each dissection was manually counted and recorded for each replicate and the dietary condition. After calculating the average values for treatment groups and (s.e.m) in Excel, data analysis was conducted using one-way ANOVA and Tukey post-hoc tests in JASP.

Caterpillar MTs: Transepithelial Potential and Basolateral Membrane Potential of Secretory Principal Cells

Dissection of the MTs from larval *Trichoplusia ni* which were raised on two different diets: control or high-water, was performed as described above (see *Caterpillar MTs Morphometrics: Secondary Cell Count*). Six control and six high-water larvae were dissected out to measure transepithelial membrane potential. In addition, ten control larval and five high water were dissected to measure basolateral membrane potential.

A microdissection of the larval *Trichoplusia ni* MTs was used to record membrane potential (Vm) from the ileac plexus using electrophysiology. To infer if there is a change in the overall transport of ions by the MTs depending on the caterpillar diet, transepithelial Vm (Vte) was measured. Glass microelectrodes were pulled using a microelectrode puller PUL-1000 (WPI Instruments), back-filled with 150 mM KCl salt solution, and mounted on a chloride silver wire electrode holder of an electrophysiology rig consisting of a PowerLab 2/26 coupled with a pH pod and inserted either into the MTs lumen (Vte) tubule lumen or into a secretory principal cell, measuring basolateral membrane potential (Vbl). Agar-salt solution-filled reference electrode was placed in the bath and connected to the rig. Manual micromanipulators were used to position the recording electrode near an epithelial cell, (a secondary cell for Vte measurement, or a principal cell for Vbl measurements). The microelectrode was inserted either into the lumen (Vte) or into a principal cell (measuring Vbl). Petri dishes coated with poly-L-lysine were employed to prevent tubule movement during electrode insertion, securing the tubule to the dish bottom. Once the microelectrode was inside the cell or the MTs lumen, electricity flowed from the recording electrode to the reference electrode, encountering resistance from cell membranes and cell-cell junctions between adjacent epithelial cells. To ensure stable and accurate readings, "shuffle" noise and movement surrounding the rig were minimized, while the rigs were assembled (prior to dissection and recording) on pneumatic isolation anti-vibration platforms. To translate this subtle tissue signal into a computer-readable format, a pre-amp (pH pod) and amplifier (PowerLab 2/26) were used to magnify and digitize the analog electrical signal captured by the microelectrodes. The microelectrodes were connected to the pre-amp via a chemically chloride silver wire inserted into the microelectrode back-filled with salt solution, electrically linking the tissue to the hardware. After calculating the average values for treatment groups and their respective s.e.m. in Excel, statistical data analysis was conducted using JASP using Student's t-test.

Cricket MTs: Functional Dye Accumulation Bioassay

To commence, Malpighian tubules of crickets were dissected out (n=34 in total) and left attached to the gut. Before conducting pharmacological experiments, one cricket was dissected and its MTs incubated in chlorophenol red (CPR) to confirm that a measurable assay could be run and that the dye accumulated in the lumen of the tubules. The head, legs, and wings of the cricket were cut off after cricket anesthesia. In the dissecting dish, the cricket was placed in a ventral up position and pinned through the thorax. The last abdominal segment was cut open followed by two cuts on each side of the abdomen. Internal organs were exposed when the ventral segment was peeled off. The gut was confirmed to still be intact, and the MTs were gently lifted out while attached to the gut. If either the MTs or the gut were visibly damaged, the prep was discarded, and the dissection was carried out anew. Each set of MTs was immersed in saline solution while dissecting and before it was ready for experimentation. Then, the saline was withdrawn using a transfer pipet and replaced with one of the designated test solutions: CPR (control), CPR with dinitrophenol (CPR+DNP), CPR with dinitrophenol supplemented with ATP (CPR+DNP+ATP), CPR with ouabain (CPR+Ouabain), or Na^+-free CPR solution, all of which had been prepared prior to the laboratory session by the instructional support

technician team. Following the addition of the solutions, the MTs preps were allowed to incubate at room temperature for a duration of 10 min. At the end of the incubation period, the MTs preps were rinsed with fresh saline without CPR, and were then subjected to luminal dye accumulation color grading. A scale ranging from 0 to 5 was used to measure CPR dye accumulation in the lumen, with 5 indicating the highest concentration, assigned automatically to the CPR (control) group. Data were tabulated in Excel, where averages and standard error of the mean (s.e.m.) were calculated, and data analysis was performed to identify which pharmacological agents significantly altered dye accumulation, and thus the excretory function, of the cricket MTs using JASP and a combination of a one-way ANOVA and a series of multiple-comparison Tukey post-hoc tests.

Morphometrics of Anal Papillae in the Larval Mosquito: Length and Width Measurements

Larvae of *Aedes aegypti* were raised from hatched eggs in environments of the following salinities - 0ppt, 3ppt, 6ppt, and 9ppt (prepared using commercially available Instant Ocean salts) to test the phenotypic plasticity of anal papillae based on availability of environmental ions. A total of nine larvae were analyzed for each salinity group. The larvae were gently captured using a transfer pipet from the growing chambers and placed on a Kim wipe to lay flat. A microscope with a calibrated ocular micrometer was used to measure body length, as well as anal papilla length and width. To standardize the measurements, anal papillae length was divided by the body length of the respective larvae to account for overall differences in animal size. Standardization was repeated for width measurements of anal papillae. Calculated ratios of papilla length/body length and papilla width/body length were tabulated in Excel, where averages and s.e.m. were calculated for each parameter and treatment group. Data analysis was conducted using one-way ANOVA and Tukey post-hoc tests in JASP.

Results

To investigate whether environmental salt and water balance will alter the hydromineral balance of insects, as well as the structure and function of osmoregulatory organs, we subjected caterpillars and mosquito larvae to dietary and environmental salt and water imbalance. Additionally, we used MTs of crickets to demonstrate that active ion transport, Na^+ gradient, and Na^+/K^+-ATPase are used to generate fluid secretion and urine formation by the MTs.

To investigate whether the effect of dietary ion content may pose an impact on the ion concentration in the caterpillar hemolymph, we measured hemolymph osmolality under two different dietary conditions; a control diet and a high-K^+ diet. Average osmolality for the control groups was 414±15, while in the high-K^+ group, it was 448±17. A t-test determined that there was no significant difference in hemolymph serum osmolality between two groups (**Fig 1**) (Student t- test, p=0.149).

Secondary cells of MTs in caterpillars (*Trichoplusia ni*) were counted under control, high- ion, and high-water diets to investigate the abundance of epithelial cells and their connection to different ion-transporting phenotypes in MTs. A total of 28 *Trichoplusia ni* larvae were dissected for the study. Nine *Trichoplusia ni* in the control group had a mean value of 10.78 ± 0.85 secondary cells manually counted under a dissection microscope in Figure 3. In the high ion diet, 12 *Trichoplusia ni* had a secondary cell count with a mean value of 10.58 ± 1.10 (**Fig 2**). The high- water diet had a total of seven *Trichoplusia ni* with a secondary cell count mean value of 6 ± 0.76 (**Fig 2**). A one-way ANOVA was run to determine a difference of means in the three experimental trials. A significant difference was found with p-value of 0.007 (**Table 1**). Following the ANOVA, a post hoc Tukey mean separations test was performed to determine significant differences between experimental diet groups. A significant difference was found between the control and high ion diets (p =

0.013) as well as between the high ion and high-water diets (p = 0.012) (**Table 1**). There was no significant difference found between the high ion and control groups with a p-value of 0.989 (**Table 1**).

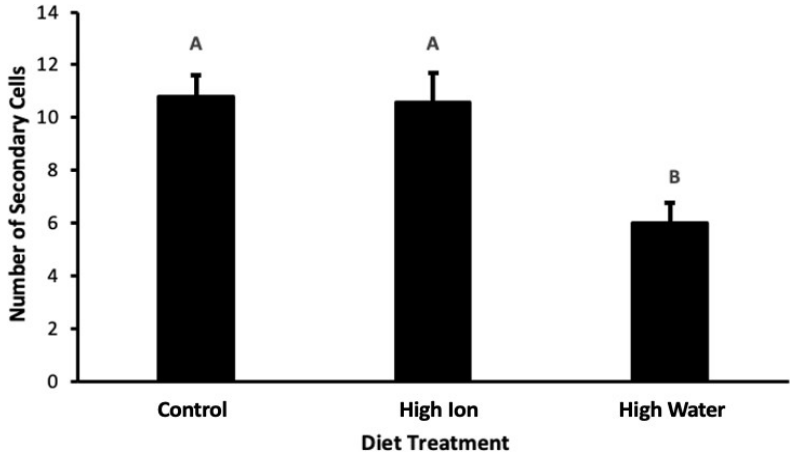

Figure 1. Average hemolymph serum osmolality of larval *Trichoplusia ni* raised on two different diets - control and high potassium. Average caterpillar osmolality ± standard error mean for the control groups was 414 ± 15. For the high potassium group, 448 ± 17.

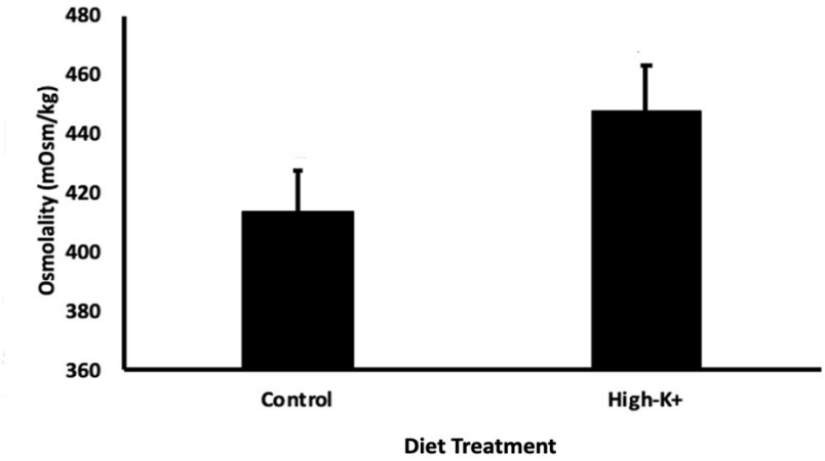

Figure 2. The mean number of secondary cells in the control group was 10.78 ± .85. In the high- ion diet, the mean number of secondary cells counted was 10.58 ± 1.10. The high-water diet had a mean value of 6 ± 0.76. A one-way ANOVA was run, and a significant difference was found with p-value 0.007.

Two different diets of caterpillar larvae (*Trichoplusia ni*) underwent measurements for their transepithelial membrane (Vte) and basolateral membrane (Vbl) to understand the action potential level depending on the diet. The bar graph illustrates the transepithelial membrane (Vte) in different diets (control vs high H2O) (**Fig 3**). The high-H2O diet of *Trichoplusia ni* appeared to have a greater epithelial membrane potential compared to control (**Fig 3A**). The independent t-test however, proved no significant difference between control and high-H2O since the p-value > 0.05 (p- value=0.138) (**Table 2**). Juxtapose, the bar graph presented the basolateral membrane (Vbl) with a different diet opposite to the control and showed a larger epithelial membrane potential compared to the high- H2O diet (**Fig 3B**). There was no significant difference found between the two diets since the p-value > 0.05 (p-value=0.603) (**Table 3**).

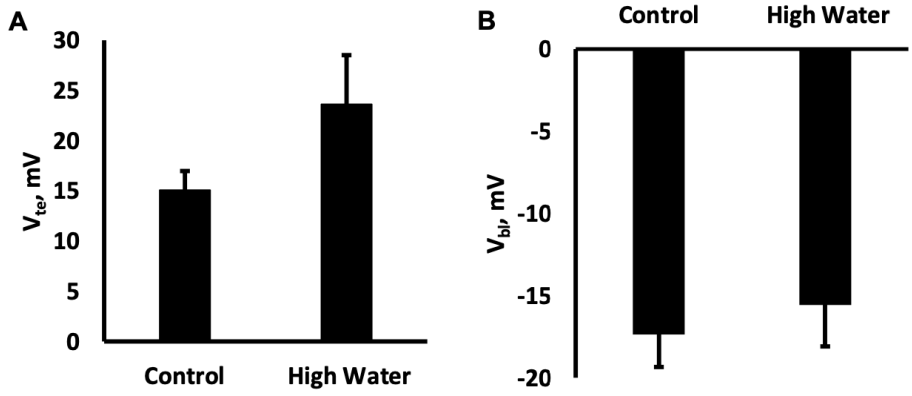

Figure 3. (A) Transepithelial membrane potential (V_{te}) and (B) basolateral membrane potential (Vbl) in the Malpighian tubule cells of the control group and in the high-water group of *Trichoplusia ni*.

Adult crickets were used to investigate whether the Malpighian tubules employ active ion transport mechanisms and whether they specifically employ Na^+/K^+-ATPase, which builds a Na^+ ion gradient to excrete chlorophenol red (CPR), an organic anion dye. The control group demonstrated the highest dye accumulation score as a reference to measure absorption of cricket Malpighian tubules. The average score of dye accumulation into Malpighian tubules was 2.286 ± 0.36 for the group treated with DNP, 1.714 ± 0.474 for the group treated with DNP and ATP, 3.286 ± 0.421 for CPR/ Na^+-Free, and 2.714 ± 0.286 for CPR/ouabain (**Fig 4**). The average ranking for CPR/ Na^+-Free was significantly higher than the average for CPR/DNP/ATP with a p-value less than 0.05 (**Fig 2**). The average ranking of the control group CPR was significantly higher than the average ranking of all four treatment groups (p-value <0.05) (**Fig 4**).

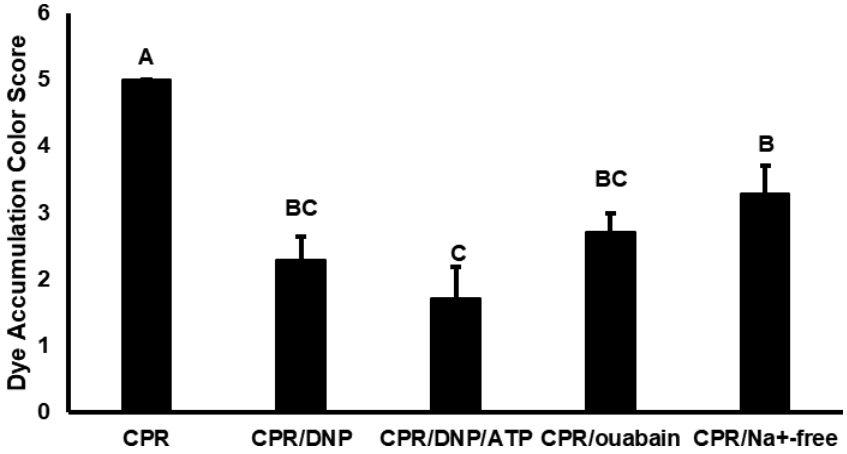

Figure 4. Dye accumulation in the Malpighian tubules of *Acheta domesticus* after a 10-minute incubation period as a quick measure of Malpighian tubule function. Mean concentration score ± s.e.m. Upper case letters are used to indicate a statistically significant differences of the means

The anal papillae of mosquito larvae (*A. aegypti*) were measured under different sodium concentrations to assess the impact on their length and width. The length of anal papillae decreased in treatment groups with high salinity (**Fig 5A**). The mean ratio of anal papillae to body length at concentrations of 0 parts per thousand (ppt) had a mean length ratio of 0.203 ± 0.022 (**Fig 5A**). At 3ppt they had a mean length ratio of 0.133 ± 0.009 (**Fig 5A**). For the group at 6ppt they had a mean ratio of 0.166 ± 0.013 and the group at a sodium concentration of 9ppt had a mean length ratio of 0.127 ± 0.018 (**Fig 5A**). The length of the anal papillae at 0 ppt of sodium were significantly different from 3ppt (p=0.027) and 9ppt

(p=0.014), with 3 and 9 ppt concentrations not being significantly different (p=0.993) (**Fig 5A**). The length of the anal papillae from larvae grown in 6ppt of sodium was not significantly different from 0 ppt (p=0.391). Furthermore, the length of anal papillae in larvae grown in 6ppt of sodium were not significantly different 3 (p=0.528) and 9 ppt (p=0.375) (**Fig 5A**).

The width of the anal papillae by total larval length in a sodium concentration of 0ppt had a ratio of 0.0406 ±0.0047 (**Fig 5B**). In a higher sodium environment with a concentration of 3ppt there was an average anal papillae width per total length of 0.0352 ±0.0035 (**Fig 5B**). In an environment with a higher sodium concentration of 6ppt there was an average anal papillae width per length ratio of 0.0380 ±0.0030 (**Fig 5B**). Lastly in a high concentration of sodium with a concentration of 9ppt there was an average anal papillae width per total length ratio of 0.0373 ± 0.0033 (**Fig 5B**). An ANOVA was performed and found the widths of anal papillae at different sodium concentrations were not statistically different with a p-value greater than 0.05 (**Fig 5B**).

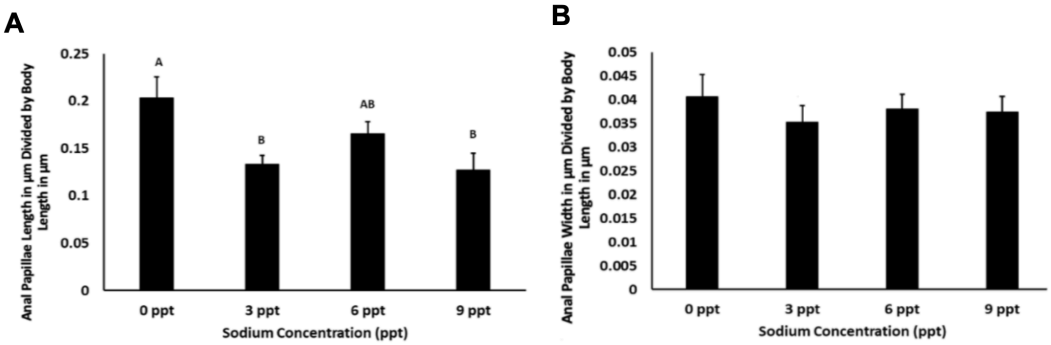

Figure 5. Morphometrics of mosquito larva (*Ae. aegypti*) anal papillae in the rearing environments of varying salt concentrations in parts per thousand (ppt): (A) papilla length/body length ratios, and (B) papilla width/body width ratios. (A) A one-way ANOVA found that anal papillae lengths at 0ppt were statistically different from 3 ppt (p=0.027) and 9 ppt (p=0.014). The larva in a sodium concentration of 6 ppt was not found to have a statistically significant difference from 0 ppt (p=0.391), or from 3 ppt (p=0.528) or 9 ppt (p=0.375). (B) No difference in papilla width/body length ratios were found between any of the treatment groups.

Discussion

Overall, the focus of this series of experiments was to examine how osmoregulation in insects respond when we alter ion availability in their diet or in their environment, and to evaluate how their osmoregulatory tissues change in structure and function in response to that. Overall, we have found the insects to be resilient and demonstrate altered osmoregulatory organ morphometrics to maintain the organ function and ensure that overall salt and water balance is not perturbed. For instance, caterpillars would decrease the numbers of reabsorptive secondary cells when fed ion- poor high-water diet, without altering overall ion transport in the MTs. In contrast, high-ion diets did not perturb the structure of the function of MTs, and did not result in systemic changes of ion levels in the hemolymph of the larvae. We found that Na+/K+-ATPase and Na+ channels are likely used in the MTs of crickets to generate fluid secreted by this osmoregulatory organ. Lastly, we found that although mosquito larvae decrease the length (but not width) of anal papillae in ion-rich environments, they do not do so indefinitely no matter the salinity increase.

Serum osmolality refers to the concentration of all dissolved particles in the blood including ions and waste that are processed within an insect's circulatory system.

Osmoregulation and nutrient absorption aid insect growth cycles and contribute to insect fecundity and success. Abiotic factors, such as increased or decreased availability of ions and water, can impact variables such as water intake and loss, which in turn directly impact hemolymph serum osmolality of the insect. Examining serum osmolality in caterpillars provided insights into ion concentration, revealing the impact of dietary ion availability on the insect's blood composition. It was hypothesized that the overall osmolality would increase once K^+ is added to the diet. Given that we found no significant difference in serum osmolality between larvae raised on different diets, increasing the dietary concentration of K^+ (one of the primary osmolytes of plant-eating insects) indicated that the larvae likely increased K^+ loss through diuresis, or supplemented acquisition of additional K^+ with additional water via osmosis, resulting in unperturbed serum osmolality. In general, in insects, increasing the amount of K^+ in the diet does impact the serum osmolality because it accelerates the transport of additional ions that are present in the luminal surface of the epithelium layer on the MTs (Anderson & Harvey, 1966; Blankemeyer & Harvey, 1978; Thomas & May, 1983). In addition, previous studies in larval *T. ni*, however, found increased hemolymph [K^+] in larvae exposed to high-K^+ diet (Kolosov et al, 2018b). Perhaps, a higher sample size would reveal this difference, given that the observed values in the current study are trending towards increased osmolality and nearing significance.

Restating the initial hypothesis, when an individual caterpillar has more ions naturally absorbed from their diets, it is thought that they would have more secondary cells located in the MTs, since secondary cells are reabsorptive and the MTs in larvae raised of larvae raised on high- K^+ diet reabsorb more K^+ via secondary cells (Kolosov et al, 2018a; Kolosov et al, 2018b). However, we found no differences in secondary cell numbers in the MTs of larvae raised on high- K^+ diet compared to control diets. The purpose of secondary cells is to reabsorb cations into the hemolymph, such as K^+ and Na^+, to maintain homeostasis. This likely means that while larvae increase the rate of K^+ reabsorption via individual secondary cells, they do not increase secondary cell numbers to compound this effect.

In contrast, when caterpillars were raised on a high-water diet (where additional water diluted available ions, and thus could be treated as ion-poor diet), our results showed that the caterpillars had fewer secondary cells in the MTs. This would agree with previous studies that demonstrate that in larvae raised on low-ion diets less ion reabsorption happens in their MTs, and less ions are reabsorbed specifically via secondary cells (Kolosov et al, 2018a; Kolosov et al, 2018b). Additionally, an experiment performed by O'Donnell and Ruiz-Sanchez targeted the reabsorption rates of ions in larvae with full guts and empty guts demonstrated that secondary cells were shown to have an increase in absorption of K^+ in larvae with full guts (O'Donnell and Sanchez, 2015). So, the MTs of larvae with empty guts or with diets with fewer ions would reabsorb ions less and would presumably have less use for secondary cells. Thus, decreased secondary cell counts in the MTs of larvae that exhibit a more secretory phenotype is not unexpected. What this means for the larvae, is that it can easily acclimate to ion-rich diets without changing the structure and function of its MTs, while ion-poor environments may prove more challenging requiring cellular restructuring of the MTs.

Measuring membrane potential (Vm) in epithelial cells helps investigate the total ion permeability and ion transport of the MTs of insects. Changes in ion transport and permeability alter Vm due to ion pumps (e.g., Na^+/K^+-ATPase), transporters and channels working based on the available ion concertation (O'Donnell, 2008). Comparing membrane potential between control and high-water groups in both Vte and Vbl membrane potentials demonstrated no significant differences. This does not support our hypothesis as we hypothesized there would be a significant decrease in both parameters because of the low

presence of ions in the high-water diet, which typically leads to a more secretory MTs phenotype. More ion secretion would typically result in more polarized Vm values. This could be attributed to caterpillars acclimating to the lack of ions, allowing them to achieve homeostasis in conditions corresponding by lowering the number of secondary cells in MTs when raised on a high-water diet without adjusting the ion transport in individual cells. A recent review supported the evidence that a caterpillar MTs are extremely efficient in maintaining a constant internal environment (Dow et al., 2021). Thus, lending support to our findings that while the MTs of caterpillars demonstrated slightly higher V_{te} in the high-water diet, there is no statistical significance from the control diet, where, perhaps, increased replicate numbers would again help discern whether the changes trending towards significance are real. Further experimentation with larger sample sizes dissected immediately after a meal and varied high-ion treatment groups is necessary for comprehensive analysis.

When incubated with CPR, cricket Malpighian tubules actively secrete the dye into the lumen against a concentration gradient, the way they' excrete any other metabolic waste. Therefore, the accumulation of the dye can be used to estimate how well the MTs are functioning. In the presence of DNP, a decrease in mitochondrial ATP production is caused by the DNP being a mitochondrial uncoupler agent, which removes the hydrogen ions that create the outer membrane gradient, into the mitochondrial matrix (Geisler et al., 2017). This results in a decreased production of ATP, leading to decreased primary and secondary active ion transport in the MTs. The addition of DNP will cause a loss of active transport, but CPR would still be able to diffuse passively via cell-cell junction, therefore some dye accumulation was expected. The average dye accumulation score for the CPR/DNP treatment group supports the hypothesis that DNP would significantly inhibit the ion transport and fluid secretion of the MTs. The lower score of CPR/DNP/ATP when compared to CPR/DNP was not expected. The external addition of ATP was anticipated to act as a form of rescue for the loss of active transport which would result in a higher dye accumulation score than CPR/DNP. The lower dye accumulation of CPR/DNP/ATP compared to CPR/DNP may be a result of an insufficient amount of external ATP added. If not enough ATP was added to counterbalance the effects of DNP, then the MTs function would remain low as observed. The addition of a specific Na^+/K^+-ATPase inhibitor ouabain inhibited the ion pump, causing a decrease in the generated Na^+ gradient. As a result, indirect active transport relying on the Na^+ gradient was unable to function. Organic anions such as CPR (and metabolic wasted transported by similar transporters in the MTs) depend on indirect active transport coupled to the Na^+ gradient, therefore the presence of ouabain resulted in an expected decrease in dye accumulation as it slowed diffusion across the lumen (Maddrell and Overton, 1988). A previous study investigating the relationship between the intracellular ATP concentration and the electrical properties of principal cells found that the addition of DNP caused the intracellular ATP concentration to decrease to values that were below 10% of the control value (Wu & Beyenbach, 2003). Alternatively, inhibiting Na^+ transport using Na^+-free CPR saline was only directed towards Na^+ channel, which was shown to result in lower dye accumulation compared to control CPR solution. Analyzing the relative effect of pharmacological inhibitors as well as Na^+-free saline allowed us to confirm that Na^+/K^+-ATPase and Na^+ channels, as well as waste transport through the cell-cell junctions is used to generated fluid secreted by the cricket MTs.

The anal papillae of mosquito larvae in both freshwater and brackish water environments, play a crucial role in maintaining proper Na^+ and Cl^- balance (Durant et al., 2021). Increased length of anal papillae in the larval *Ae. aegypti* revealed the phenotypic plasticity of the species as it acclimated to different environmental salinity levels. The anal

papillae of *Ae. aegypti* decrease in surface and volume to limit the active uptake of so Na^+ and Cl^- in brackish water environments (>0 ppt) while they become larger to promote absorption of ions in freshwater environments (0 ppt). Normalized length of anal papillae in mosquito larvae of the current study was significantly decreased in groups raised in brackish water habitats. However, further decrease in size of anal papillae in more saline environments (9 ppt compared to 6 ppt; 6 ppt compared to 3 ppt, etc.) was limited by other crucial roles of anal papillae such as ammonia excretion.

Each anal papillae is a syncytium composed of multiple cells and are cylindrical in structure, meaning they effectively shrink in length however do not reduce in width (Edwards & Harrison, 1983). This agrees with observations of no difference in normalized anal papilla width between any treatment groups in the current study. The overall results indicate that the other roles of anal papillae, outside of salt and water balance, outweigh the benefits of limiting salt absorption in high-salinity environments. Understanding how anal papillae acclimate and function to allow mosquito larvae to thrive in various water sources is important in figuring out ways to limit the transmission of infectious diseases by adult mosquitoes that develop from mosquito larvae. Previous studies have shown that anal papillae in mosquito larvae are thought to be controlled by endocrine signaling (Edwards & Harrison, 1983). Therefore, there is potential for creating an effective insecticide that would prevent the anal papillae from changing in size with salinity acclimation resulting in larval death.

Understanding how dietary and environmental ion availability influences terrestrial and aquatic insects involves examining the impact of ion and water concentrations on the prime osmoregulatory organs like MTs and anal papillae, as well as examining the molecular mechanisms of epithelial ion transport in insect MTs. By closely investigating how excretory systems react to various stressors, we can contribute to the foundational knowledge of osmoregulation in this economically and ecologically important group of diverse invertebrates. Pesticides target specific insects but can sometimes inadvertently harm beneficial ones. Mosquitoes are vectors for many deadly diseases that impact our health, while caterpillars can damage crops, negatively affecting our agricultural economy. By investigating unique osmoregulatory and excretion systems of each insect, we can develop species-specific insecticides that effectively target harmful insects like mosquitoes and caterpillars, while leaving beneficial insects like pollinators unaffected. Thus, the right pesticide should not pose harm to beneficial pollinators and populations of other animals, while contributing to a healthier ecosystem as our relationships with insects can be maintained at a peaceful homeostasis.

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Appendix: Statistical test results

Table 1. One-way ANOVA was performed with the mean number of secondary cells for each diet as the variables. A significant difference was determined with a p-value 0.007 (<0.05). A post hoc Tukey's test was used to compare each experimental group and a significant difference was determined between control and high-water diets as well as high ion and high-water diets.

ANOVA- Secondary Cells					
Cases	Sum of Squares	df	Mean Square	F	p
Groups	114.528	2	57.264	6.054	0.007
Residuals	236.472	25	9.459		
Note. Type III Sum of Squares					

Post Hoc Comparisons-Groups					
		Mean Differences	SE	t	p_{tukey}
Control	High Ion	0.194	1.356	0.143	0.989
High Water		4.778	1.550	3.083	0.013
High Ion	High Water	4.583	1.463	3.133	0.012
Note. P-value adjusted for comparing a family of 3					

Table 2: Independent T-test of the total transepithelial membrane potential (mV) between the control group and in the High H2O group of *Trichoplusia ni*. Shapiro-Wilk and Levene's test was followed. No significant difference has been shown between the two different caterpillar larvae diets (control and high H2O) since the p-value is greater than 0.05.

V_{te} Independent Samples T-Test

Independent Samples T-Test

		Statistic	df	p
Vte data	Student's t	-1.61	10.0	0.138

Note. H_a μ control ≠ μ High H2O

Assumptions

Normality Test (Shapiro-Wilk)

	W	p
Vte data	0.904	0.181

Note. A low p-value suggests a violation of the assumption of normality

Homogeneity of Variances Test (Levene's)

	F	df	df2	p
Vte data	4.23	1	10	0.067

Note. A low p-value suggests a violation of the assumption of equal variances

Table 3: Independent T-test of the basolateral membrane (mV) between the control group and in the High H2O group of *Trichoplusia ni*. Shapiro-Wilk and Levene's test was followed. No significant difference has been shown between the two different caterpillar larvae diets (control and high-H2O) since the p-value is >0.05.

Vbl Independent Samples T-Test

Independent Samples T-Test

		Statistic	df	p
Vbl data	Student's t	-0.532	13.0	0.603

Note. H_a μ control ≠ μ High H2O

Assumptions

Normality Test (Shapiro-Wilk)

	W	p
Vbl data	0.871	0.034

Note. A low p-value suggests a violation of the assumption of normality

Homogeneity of Variances Test (Levene's)

	F	df	df2	p
Vbl data	0.565	1	13	0.466

Note. A low p-value suggests a violation of the assumption of equal variances