Amino Acid Substitutions in the Na^+/K^+-ATPase May Contribute to Salinity Tolerance in Insects

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Abstract

1. **Environmental salinity levels vary naturally across terrestrial ecosystems but can be heightened locally by coastal proximity and desertification as well as human activities such as road salt application and agriculture. Since salt is essential for many physiological processes in insects, rising environmental sodium concentrations may drive behavioral changes, where insects select environments and food sources with suitable sodium levels, or evolutionary changes in constitutive or plastic physiological mechanisms to process salt, potentially altering ecological dynamics and species interactions.**
2. **Numerous hematophagous (blood feeding) insects such as the yellow-fever mosquito *Aedes aeqypti* are known to be able to breed in relatively saline environments. Among phytophagous (plant feeding) insects, grasshoppers can be important herbivores in arid and coastal salt-affected regions, whereas the monarch butterfly**

(*Danaus plexippus*) appears to perform relatively well on milkweed host plants growing in roadsides influenced by salt runoff. Several of these insects share a common trait: amino acid substitutions in the first extracellular loop of the Na^+/K^+-ATPase (NKA), a sodium pump crucial for maintaining ion balance. For the monarch these substitutions confer resistance to toxic cardenolides from milkweeds, but it is unclear whether NKA substitutions may influence salt tolerance.

3. **Here, we investigate whether the NKA substitutions found in these insects may contribute to salt tolerance using gene-edited *Drosophila melanogaster* mutant strains as models. We show that flies with substitution Q111L (found in *Aedes* mosquitoes) or a combination of Q111L and A119S (found in grasshoppers) exhibited greater salt tolerance, whereas flies carrying the combination of substitutions found in the monarch (Q111V, A119S, and N122H) did not.**
4. **Our results suggest that the monarch may rely on alternate mechanisms for salt tolerance and that its NKA substitutions are important primarily for cardenolide resistance. However, substitution Q111L and the combination of Q111L and A119S may be relevant for salt tolerance in a variety of insects. Uncovering mechanisms of salt tolerance enhances our understanding of species distributions, ecological interactions, and evolutionary physiology in response to changing environmental salinity levels.**

Introduction

Salinity levels vary geographically across terrestrial ecosystems, particularly in coastal regions (Lorrain-Soligon et al., 2023). Sodium levels in soil range from < 0.05 % (8.5 mM) to > 1.75% (300 mM) in strongly saline areas (Department of Primary Industries and Regional Development, Western Australia, 2024). Many insect species—including phytophagous, hematophagous, and aquatic insects—may thus encounter a wide range of salt concentrations across their habitats (Santiago-Rosario et al., 2025). Sodium (Na^+), while generally non-essential for most plants, is a key nutrient for animals to support development and physiological processes. Insects have therefore evolved several behavioral and physiological adaptations to not only ensure adequate sodium intake but also to process excess salt, as it may become toxic at levels higher than a physiological level of c. 0.9% (154 mM; Snell-Rood et al., 2014; Pontes et

al., 2022; Santiago-Rosario et al., 2022). As an example of behavioral adaptation, butterflies engage in puddling, where they extract sodium from soil to compensate for the relatively low salt contents of their host plants, which is often < 0.05% (Xiao et al., 2010; Snell-Rood et al., 2014). This behavior is essential for supporting nervous system functions, growth, and reproductive processes (Lamie et al., 2025). Interestingly, plants may use sodium withholding as an anti-herbivore defense mechanism since they do not require high sodium for metabolic functions (Kaspari, 2020). On the other hand, following the "No-Escape-from-Sodium" hypothesis, sodium concentrations of plant tissues tend to reflect environmental salt levels (Santiago-Rosario et al., 2022). Sodium concentrations of salt-tolerant plants such as *Atriplex* saltbushes and salt-accumulating species such as the salt marsh grass *Spartina alterniflora* can reach well above physiological levels for animals (Santiago-Rosario et al., 2022; Vasquez et al., 2006). As such, environmental salt levels influence the phytochemical landscape, which in turn may affect interactions with phytophagous animals and even the structure of ecological communities (Santiago-Rosario et al., 2022).

Insect adaptations to fluctuating salt levels could become more important now that salinity levels are increasing to above physiological levels for animals across large areas due to climate change-associated desertification and rising sea levels as well as human activities such as road salt application and agriculture (Hassani et al., 2020; Kengne et al., 2019; Snell-Rood et al., 2014). Understanding how some species can tolerate and have adapted to increases in sodium levels is crucial for explaining species' ecological distributions and interactions.

Hematophagous insects such as mosquitoes, sand flies, and stable flies form an ecologically important guild and may encounter relatively high levels of salt through blood-feeding and when breeding in saline environments (Bradley, 1987; Yee et al., 2021). For example, some representatives of the mosquito genera *Aedes*, *Anopheles*, and *Culex* such as *An. merus* can thrive in saline habitats (Kengne et al., 2019; Yee et al., 2021), while *Ae. aegypti* and *Ae. albopictus* are expanding to coastal regions and are capable of breeding in environments with moderate to high salinity levels of up to 1.5% (Ramasamy et al., 2021).

Phytophagous insects might experience high salinity if they must navigate salt-affected soils in between bouts of feeding on plants or when attacking plants growing in saline environments along the coast or at roadsides. Monarch butterflies (*Danaus plexippus*) appear to potentially

benefit from feeding as larvae on milkweeds (*Asclepias* spp.) in roadside environments (with leaf sodium concentrations closer to physiological levels for animals), since males show increased muscle mass and females exhibit greater neural investment (Snell-Rood et al., 2014; Hund et al., 2024). Both male and female monarchs accumulate relatively high sodium levels in saline environments, whereas this is only observed for males with other butterfly species (Santiago-Rosario et al., 2025). Grasshoppers, including the eastern lubber grasshopper *Romalea microptera*, can be important herbivores of plants such as *Spartina alterniflora*, which grows in coastal salt marshes and other salt-affected regions (Haines and Montague, 1979; Li et al., 2023; Vincent, 2006). Leaves of this plant can reach sodium levels of up to 3.5% (Vasquez et al., 2006). Physiological studies have shown that grasshoppers may tolerate levels of salinity reaching 11% (Dadd, 1961). Such observations raise the question of how these insects adapted to elevated environmental sodium levels.

The Na^+/K^+-ATPase (NKA) is a highly conserved sodium pump across animal species (Clausen and Poulsen, 2013). In insects, it plays a crucial role in regulating ion gradients, cell signaling, and stress responses (Groen and Whiteman, 2022). Several phytophagous and hematophagous insects that can be found in salt-affected environments, including the monarch butterfly, grasshoppers, *Aedes* mosquitoes, *Glossina* tsetse flies, *Phlebotomus* sandflies, and *Stomoxys* stable flies, evolved amino acid substitutions in the first extracellular loop of the NKA alpha subunit (Table 1; Dobler et al., 2012; Yang et al., 2019). Do these substitutions contribute to salt tolerance, given the sodium pump's function in ion regulation? For monarchs, substitutions in the NKA facilitated specialization on milkweeds, conferring resistance to toxic cardenolides through target-site insensitivity. However, it remains unclear whether these substitutions have pleiotropic effects on salt tolerance. Establishing whether NKA substitutions contribute to salt tolerance in insects that frequently encounter saline environments may provide mechanistic insights into these insects' distributions, behaviors, and ecological interactions.

We used *D. melanogaster* (Drosophila) to study gene-edited knock-in mutant strains with amino acid substitutions at sites 111, 119, and/or 122 of the NKA, reflecting substitutions found in hematophagous and phytophagous species (Groen and Whiteman, 2016; Karageorgi et al., 2019). Monarchs evolved substitutions Q111V, A119S, and N122H (VSH); *Aedes* mosquitoes evolved substitution Q111L (LAN); several hematophagous flies evolved substitution A119S (QSN); and

grasshoppers evolved both Q111L and A119S (LSN; Table 1; Dobler et al., 2012; Yang et al., 2019). Salt tolerance of Drosophila mutants was compared to a control strain with the wild-type sequence (QAN). We aimed to determine whether these NKA substitutions could influence salt tolerance in insects.

Order/Family	Species	Common Name	111	112	113	114	115	116	117	118	119	120	121	122
Orthoptera														
Tetrigidae	*Tetrix japonica*	Eastern common groundhopper	Q	A	S	T	V	E	E	P	A	D	D	N
Romaleidae	*Romalea microptera*	Eastern lubber grasshopper	L	A	S	T	V	E	E	P	S	D	D	N
Acrididae	*Locusta migratoria*	Migratory locust	L	A	A	T	V	E	E	P	S	D	D	N
	Schistocerca americana	American bird grasshopper	L	A	S	T	V	E	E	P	S	D	D	N
Diptera														
Drosophilidae	*Drosophila melanogaster*	Vinegar fly	Q	A	S	T	S	E	E	P	A	D	D	N
Glossinidae	*Glossina morsitans*	Tsetse fly	Q	A	S	T	S	E	E	P	S	D	D	N
Muscidae	*Stomoxys calcitrans*	Stable fly	Q	A	S	T	S	E	E	P	S	D	D	N
Culicidae	*Culex pipiens*	Common house mosquito	Q	A	S	T	V	E	E	P	A	D	D	N
	Culex quinquefasciatus	Southern house mosquito	Q	A	S	T	V	E	E	P	A	D	D	N
	Aedes aegypti	Yellow fever mosquito	L	A	S	T	V	E	E	P	A	D	D	N
	Aedes albopictus	Asian tiger mosquito	L	A	S	T	V	E	E	P	A	D	D	N
	Anopheles coluzzii	Malaria mosquito	Q	A	S	T	V	E	E	P	A	D	D	N
	Anopheles gambiae	Malaria mosquito	Q	A	S	T	V	E	E	P	A	D	D	N
Psychodidae	*Phlebotomus papatasi*	Sand fly	Q	A	S	T	S	E	E	P	S	D	D	N
Lepidoptera														
Pieridae	*Pieris rapae*	Small cabbage white	Q	A	S	T	V	E	E	P	A	D	D	N
	Danaus plexippus	Monarch butterfly	V	A	S	T	V	E	E	P	S	D	D	H
Study model														
Drosophilidae	*Drosophila melanogaster*	QAN	Q	A	S	T	S	E	E	P	A	D	D	N
		LAN	L	A	S	T	S	E	E	P	A	D	D	N
		QSN	Q	A	S	T	S	E	E	P	S	D	D	N
		LSN	L	A	S	T	S	E	E	P	S	D	D	N
		VSH	V	A	S	T	S	E	E	P	S	D	D	H

Table 1. Amino acid sequences of the Na^+/K^+-ATPases of insects in the orders Orthoptera, Diptera, and Lepidoptera that have been found to show variation in their levels of salt tolerance. Sequences of the Na^+/K^+-ATPases of our study model Drosophila (*D. melanogaster*) are also given for the wild type (QAN) and gene-edited knock-in mutant strains (QSN, LAN, LSN, and VSH). The sequences at positions 111–122 of the Na^+/K^+-ATPase alpha subunit are shown (numbering relative to the pig ATP1A1 sequence).

Methods

Drosophila maintenance and culturing. Drosophila strains [QAN], [LAN], [QSN], [LSN], and [VSH] with or without substitutions at positions 111, 119, and/or 122 of the NKA alpha subunit in w[1118] background, were obtained from the Bloomington Drosophila Stock Center

136 (Karageorgi et al., 2019). Flies were maintained in vials with food media (Genesee Nutri-Fly) in
137 an incubator at 26 °C with a 12-hour light/dark phase.

138 **Salt tolerance assay with Drosophila.** Ten males and ten females were put into individual vials
139 containing 4 mL Genesee Nutri-Fly food for 3 days, allowing for reproduction. Ten first-instar
140 larvae were then placed into vials containing different diets; 4 mL of regular Genesee Nutri-Fly
141 media or media containing an additional 2% or 3% NaCl, which was mixed in before heating
142 during food preparation. Wild-type larvae did not survive on food containing ≥5% NaCl. Three
143 to five vials, containing ten first-instar larvae each, were used for each strain-by-diet
144 combination per trial, depending on availability of larvae. Vials were placed randomly on racks
145 with neighboring vials placed on the outer edges in an incubator with a 12-hour light/dark phase
146 at 25 °C. Data was recorded daily over a 12-day period measuring the proportions of larvae that
147 pupated and reached adulthood.

148 **Graphing and statistical analysis.** Graphs were generated as survival curves in PRISM,
149 displaying the mean and standard error of the mean (SEM). For statistical analyses
150 a binomial generalized linear model (GLM) was fitted for each developmental stage transition,
151 modeling survival (i.e., the number of individuals that survived out of the total tested) as a
152 function of strain, treatment, and their interaction using the base R statistics package (version
153 4.3.1). The R package emmeans (version 1.11.1) was used to calculate estimated marginal means
154 (EMMs) and perform post-hoc pairwise contrasts both between strains within each treatment and
155 between treatments within each strain. We further used the log-rank (LR) Mantel-Cox test to
156 perform pairwise contrasts and validate results from the GLM. Interpreting the results of these
157 analyses conservatively, results were deemed significant when both analyses indicated p values
158 <0.05. Figures were assembled using Adobe Photoshop.
159

160 ## Results

161 **Substitutions in the NKA influence salt tolerance in Drosophila.**

162 First-instar Drosophila larvae were fed diets with varying salt concentrations and monitored for
163 the proportion that pupated and eclosed (reached adulthood) successfully. We aimed to

determine whether NKA amino acid substitutions found across insects could contribute to salt tolerance (Table 1). All fly strains were significantly affected by the 2% salt treatment, with fewer individuals pupating and reaching adulthood than at 0% salt ($p<0.05$). The 3% salt treatment further exacerbated these patterns for each strain ($p<0.05$).

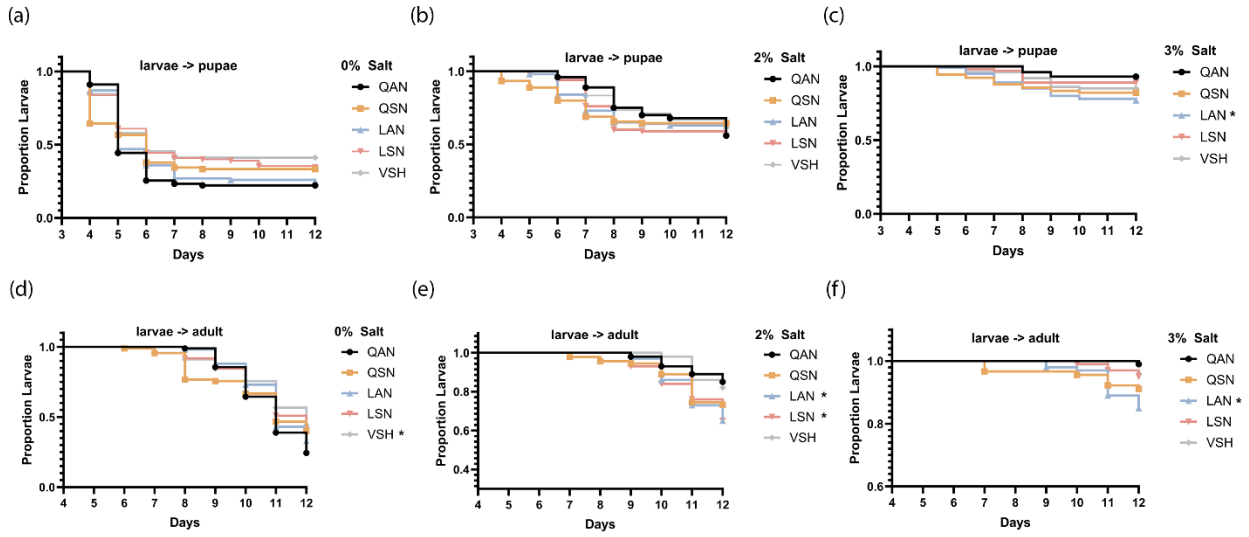

Fig. 1 The LAN and LSN knock-in mutant Drosophila strains demonstrate higher salt tolerance by their increased ability of larvae to reach the pupal and adult stages. **A-C**. Proportion of first-instar Drosophila larvae of the QAN (wild type), QSN, LAN, LSN, and VSH strains that developed into pupae after being reared on diets containing 0%, 2% or 3% salt, respectively, over a 12-day period. **D-F**. Proportion of first-instar Drosophila larvae of the QAN (wild type), QSN, LAN, LSN, and VSH strains that successfully reached the adult stage post pupation after being reared on diets containing 0%, 2% or 3% salt, respectively, over a 12-day period. Data are represented as means ± SEM. Statistical analyses were performed using log-rank analysis and an analysis using a binomial generalized linear model with pairwise comparisons to the QAN strain.

However, in pairwise comparisons with the control fly strain QAN, several knock-in strains were significantly less affected by saline environments (Fig. 1). Strain LAN showed no significant difference in pupation compared to QAN at 0% or 2% salt but had a higher rate of pupation in a saline environment of 3% ($p\leq0.022$). LAN began to show a significantly higher rate of eclosion than strain QAN at 2% salt and continued to show this pattern at 3% ($p\leq0.012$ and $p\leq0.048$, respectively). While strain QSN showed no significant differences in pupation or eclosion

184 relative to QAN at any of the salt concentrations tested (Fig. 1), LSN had a significantly higher
185 rate of eclosion than QAN at 2% salt ($p \leq 0.012$). VSH flies showed significantly fewer
186 individuals pupating and reaching adulthood at 0% ($p \leq 0.042$), but no other significant difference
187 was seen for VSH (Fig. 1). Taken together, our analyses found LAN to be the most salt tolerant
188 strain, with strain LSN also having a better chance of reaching adulthood at a sodium level of 2%
189 (Fig. 1).

190

191 **Discussion**

192 Using gene-edited knock-in mutant Drosophila strains as models, we tested the effects of amino
193 acid substitutions at sites 111, 119, and/or 122 in the first extracellular loop of the NKA's alpha
194 subunit—substitutions that evolved in a variety of phytophagous and hematophagous insect
195 species known to occur in salt-affected environments. We hypothesized that substitutions in the
196 sodium pump at these sites might influence salinity tolerance in insects.

197 Substitution Q111L (as observed in *Aedes* mosquitoes) or a combination of Q111L and A119S
198 (as observed in grasshoppers) enhanced salt tolerance in Drosophila, with larvae of strains LAN
199 and LSN showing higher pupation and/or eclosion rates on diets rich in salt. Previous research
200 showed that wild-type (QAN) flies had significantly fewer larvae reaching adulthood at 1.75%
201 salt and no survival at 5% salt (Riedl et al., 2016). We recapitulated these findings with diets
202 containing 2% and 3% salt and observed that LAN flies tolerated these sodium levels
203 significantly better than QAN in terms of reaching adulthood. In addition, strain LSN exhibited
204 similar patterns of higher relative salt tolerance at 2% NaCl. This suggests that substitution
205 Q111L and the combination of Q111L and A119S might improve insect salt tolerance.

206 Hematophagous insects may encounter salt in their environment in several ways, including by
207 feeding on blood, which contains 0.9% salt in humans, and through aquatic breeding in arid or
208 coastal salt-affected environments (Yee et al., 2021). The fact that *Aedes* mosquitoes evolved
209 substitution Q111L may to some extent explain *Ae. aegypti*'s and *Ae. albopictus*' successful
210 range expansions into coastal areas (Ramasamy et al., 2021). However, we note that *Anopheles*
211 and *Culex* also include species that occur in saline habitats but did not evolve Q111L (Dobler et

212 al. 2012; Kengne et al., 2019), suggesting they evolved different salt tolerance strategies. Other
213 hematophagous insects, including *Glossina* tsetse flies, *Phlebotomus* sandflies, and *Stomoxys*
214 stable flies, can similarly be found in arid or coastal saline environments and evolved
215 substitution A119S. However, since A119S did not confer enhanced salt tolerance, it is likely
216 that these species evolved alternative mechanisms as well.

217 Phytophagous insects may primarily be exposed to elevated sodium levels through feeding on
218 plants in salt-affected environments, such as roadsides and salt marshes (Snell-Rood et al.,
219 2014). Several grasshopper species are successful herbivores of plants in coastal salt marshes and
220 other salt-affected regions (Haines and Montague, 1979; Li et al., 2023; Vincent, 2006), can
221 tolerate relatively high levels of salinity (Dadd, 1961), and evolved both substitutions Q111L and
222 A119S. Our results for the Drosophila double mutant with these substitutions suggest that they
223 may contribute to salt tolerance in these herbivores. Mechanisms of salt tolerance might become
224 more important for insects since freshwater ecosystems are increasingly affected by salt
225 contamination due to factors such as climate change-related sea-level rise and desertification as
226 well as human activities, including groundwater extraction (Silver and Donini, 2021).

227 We hypothesized that insects with NKA substitutions linked to cardenolide resistance such as the
228 monarch butterfly may experience pleiotropic effects. Leaves of milkweeds growing near
229 roadways can contain elevated levels of Na^+ and sodium accumulates to relatively high titers in
230 both male and female monarch butterflies after having fed on such plants as larvae (Snell-Rood
231 et al., 2014; Santiago-Rosario et al., 2025). However, our results indicate that the combination of
232 substitutions Q111V, A119S, and N122H did not confer increased salt tolerance. This suggests
233 that milkweed-produced cardenolides were likely to be more important agents of selection
234 driving these NKA substitutions than environmental sodium (Karageorgi et al., 2019).

235 In summary, we found that substitution Q111L and the combination of Q111L and A119S may
236 contribute to salt tolerance in insects. Their evolution could have enhanced physiological
237 mechanisms for maintaining ion balance in a variety of phytophagous and hematophagous insect
238 species. These contributions to salt tolerance may offer valuable insights into how insect species
239 adapt to saline environments, influencing their ecological distributions.

240

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Data Availability

The data that support the findings of this study are openly available at Mendeley Data: https://data.mendeley.com/datasets/2n2ttnpctw/1

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