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 rising sea levels 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 are known to breed in relatively saline environments, while others such as the yellow-fever mosquito *Aedes aeqypti* are expanding their range to coastal regions. 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 highly conserved 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 the Q111L and A119S substitutions alone or in combination (found in grasshoppers and several hematophagous insects) 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, substitutions Q111L and A119S may be important 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 is essential for animal development and physiological processes, and fluctuating salt levels in environments may influence the foraging behavior of insects to ensure adequate salt intake (Snell-Rood et al., 2014; Pontes et al., 2022). For example, butterflies engage in mud-puddling, a behavior where they extract sodium from soil to compensate for low salt contents of their host plants (Xiao et al., 2010). Plants may use sodium withholding as a defense mechanism since they do not require high sodium for metabolic functions (Kaspari, 2020). Fluctuating salt levels may also drive physiological adaptations in insects, which could become more important now that salinity levels are increasing 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).

Hematophagous insects 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), while *Ae. aegypti* is expanding to coastal regions and has been found to breed in brackish environments (Ramasamy et al., 2021).

Phytophagous insects might experience high salinity if they have to navigate salt-affected soils in between bouts of feeding on plants or when feeding on plants growing in saline environments along the coast or at roadsides. Monarch butterflies (*Danaus plexippus*) appear to benefit potentially from feeding as larvae on milkweeds (*Asclepias* spp.) in roadside environments (with relatively high leaf sodium concentrations), since males show increased muscle mass and females exhibit greater neural investment (Snell-Rood et al., 2014). Several grasshopper species are herbivores in salt-affected coastal regions (Haines and Montague, 1979; Li et al., 2023). Physiological studies have shown that some grasshoppers can tolerate high levels of salinity (Dadd, 1961).

The NKA is a highly conserved sodium pump across animal species (Clausen and Poulsen, 2013). In insects, it plays a crucial role in regulating ion flow, ion gradients, cell signaling, and stress responses (Groen and Whiteman, 2022). Several phytophagous and hematophagous insects that can be found in salt-affected environments 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 *Drosophila melanogaster* (Drosophila), to study mutant strains with amino acid substitutions at sites 111, 119, and/or 122 of the NKA, reflecting substitutions found in

hematophagous and phytophagous species. 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.

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

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

Graphing and statistical analysis. Graphs were generated as survival curves in PRISM, displaying the mean and standard error of the mean (SEM). Statistical analyses were conducted using the log-rank Mantel-Cox test for pairwise comparisons between each Drosophila mutant strain and the wild-type QAN strain. Figures were assembled using Adobe Photoshop.

Results

Substitutions in the NKA influence salt tolerance in Drosophila.

First-instar Drosophila larvae were fed diets with varying salt concentrations and monitored for 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). Log-rank analysis was conducted using flies with the wild-type QAN sequence as control for pairwise comparisons. Strain QSN showed no significant difference in pupation or eclosion at 0% or 2% salt; however, at 3% salt, a higher proportion of larvae reached the pupal ($p = 0.019$) and adult ($p = 0.013$) stages (Fig. 1). Strain LAN showed no significant difference in pupation or eclosion at 0% salt but in saline environments had higher rates of pupation ($p = 0.004$ at 2%; $p = 0.001$ at 3%) and eclosion ($p = 0.006$ at 2%; $p = 0.001$ at 3%; Fig. 1). Strain LSN exhibited lower pupation and eclosion rates than QAN at 0% salt, whereas at 2% salt, a higher proportion of larvae reached adulthood ($p = 0.009$). No significant differences were observed for LSN at 3% salt (Fig. 1). VSH flies showed no significant difference in pupation at 0% salt but had marginally fewer larvae reaching adulthood ($p = 0.047$). No other significant differences were observed for VSH (Fig. 1).

Discussion

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

Mutations Q111L and A119S together and on their own enhanced salt tolerance in Drosophila, with larvae of strains LAN, QSN, and LSN showing higher pupation and/or eclosion rates on diets rich in salt. Previous research showed that wild-type (QAN) flies had significantly fewer larvae reaching adulthood at 1.75% salt and no survival at 5% salt (Riedl et al., 2016). We recapitulated these findings with 2% and 3% salt and observed that LAN, QSN, and LSN flies tolerated this better than QAN, suggesting that Q111L and A119S might improve salt tolerance.

Insects may encounter salt in their environment in several ways. Hematophagous insects feed on blood, which contains 0.9% salt in humans (Yee et al., 2021). Insects feeding on plants in salt-affected environments also experience relatively high salt exposure (Snell-Rood et al., 2014). The fact that *Aedes* mosquitoes evolved Q111L may to some extent explain *Ae. aegypti*'s successful range expansion (Ramasamy et al., 2021). However, we note that *Anopheles* and *Culex* include species that occur in saline habitats but did not evolve Q111L (Dobler et al. 2012; Kengne et al., 2019), suggesting they evolved different salt tolerance strategies. Mechanisms of salt tolerance might become increasingly important as the climate continues to change.

We hypothesized that insects with NKA substitutions linked to cardenolide resistance such as the monarch butterfly may experience pleiotropic effects. Leaves of milkweeds growing near roadways often contain higher sodium levels and sodium accumulates to relatively high levels in both male and female monarch butterflies, who could potentially benefit from this (Snell-Rood et al., 2014; Santiago-Rosario et al., 2025). However, our results indicate that the combination of substitutions Q111V, A119S, and N122H did not confer increased salt tolerance. This suggests that monarchs may rely on alternative mechanisms for salt tolerance and that milkweed cardenolides were more important agents of selection driving these NKA substitutions than environmental sodium.

In summary, we found that Q111L and A119S alone and in combination may contribute to salt tolerance. Their evolution could have enhanced physiological mechanisms for maintaining ion balance in a variety of phytophagous and hematophagous insect species. These contributions to salt tolerance could provide valuable insights into the adaptation of insect species to saline environments.

Acknowledgements

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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 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).

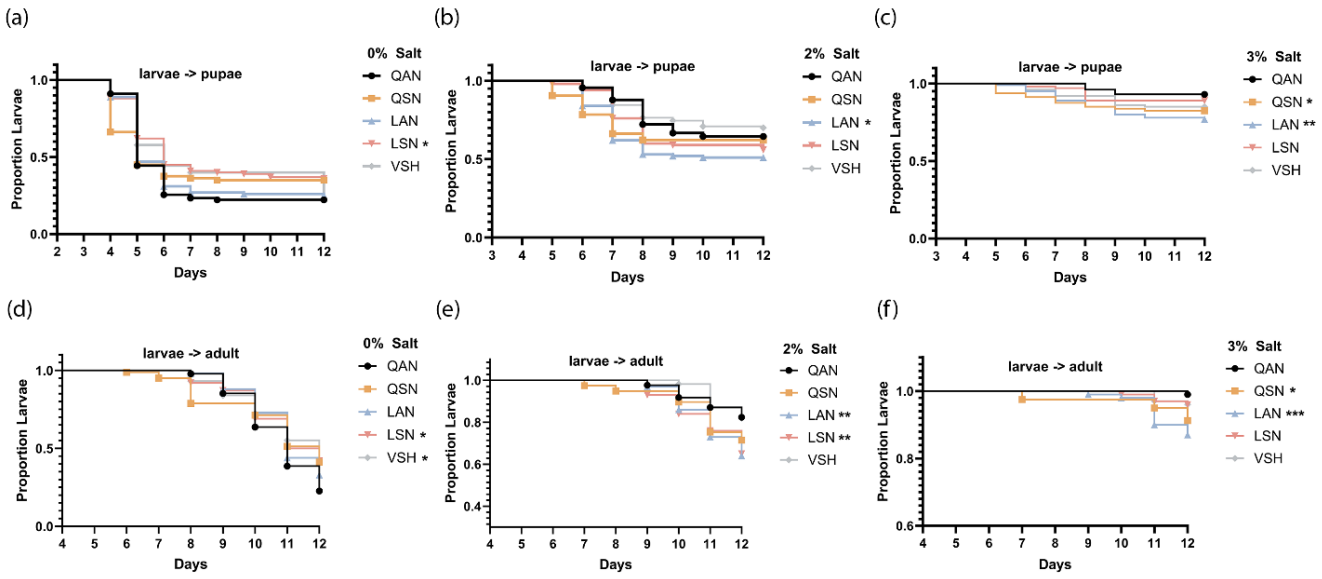

Fig. 1 The LAN, QSN, and LSN knock-in mutant Drosophila strains demonstrate higher salt tolerance by their increased ability of larvae to reach the pupal and adult stages. **a-c**. Proportion of first-instar Drosophila larvae of the QAN (wild type), QSN, LAN, and VSH strains that developed into pupae after being reared on diets containing 0%, 2% or 3% salt, respectively, over a 12-day period. **b**. Proportion of first-instar Drosophila larvae of the QAN (wild type), QSN, LAN, 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 in PRISM using log-rank analysis with pairwise comparisons to the QAN strain.

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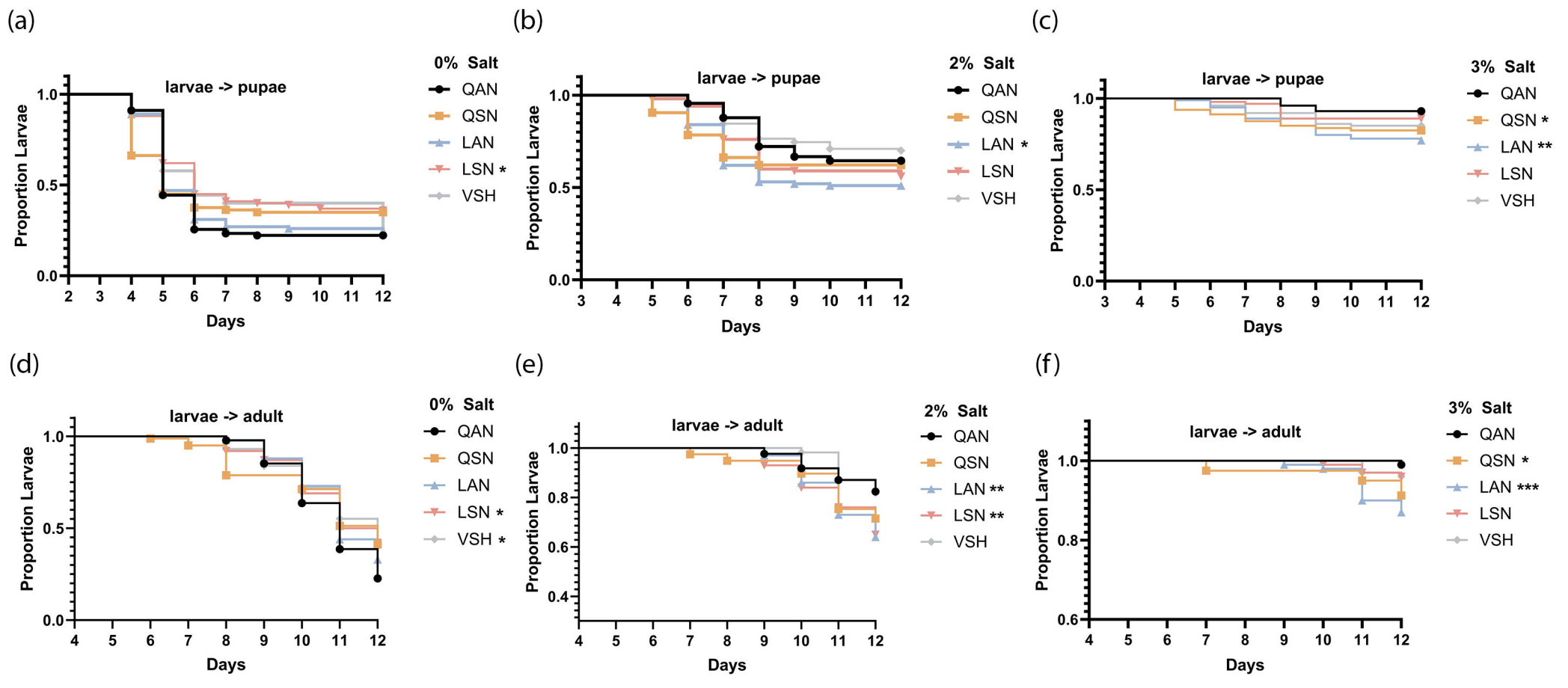
		LSN	L	A	S	T	S	E	E	P	S	D	D	N
		VSH	V	A	S	T	S	E	E	P	S	D	D	H


(a)
larvae -> pupae
Proportion Larvae
Days
0% Salt
QAN
QSN
LAN
LSN *
VSH
(b)
larvae -> pupae
Proportion Larvae
Days
2% Salt
QAN
QSN
LAN *
LSN
VSH
(c)
larvae -> pupae
Proportion Larvae
Days
3% Salt
QAN
QSN *
LAN **
LSN
VSH
(d)
larvae -> adult
Proportion Larvae
Days
0% Salt
QAN
QSN
LAN
LSN *
VSH *
(e)
larvae -> adult
Proportion Larvae
Days
2% Salt
QAN
QSN
LAN **
LSN **
VSH
(f)
larvae -> adult
Proportion Larvae
Days
3% Salt
QAN
QSN *
LAN ***
LSN
VSH