An egg is always an adventure: anthropogenic impacts on Culex pipiens population dynamics
Boerlijst, S.P.

A sample of house mosquito adults within an aspirator

Chapter 4

Taking it with a grain of salt:

tolerance to increasing salinization in Culex pipiens (Diptera: Culicidae) across a low-lying delta

Parasites & Vectors (2024)
DOI: 10.1186/s13071-024-06268-8.

Sam P. Boerlijst, Antje van der Gaast, Lisa M.W. Adema, Roderick W. Bouman, Eline Boelee, Peter M. van Bodegom and Maarten Schrama

Abstract

Salinity, exacerbated by rising sea levels, is a critical environmental cue affecting freshwater ecosystems. Predicting ecosystem structure in reaction to such changes and their implications for the geographic distribution of arthropod disease vectors requires further insights into the plasticity and adaptability of lower trophic level species in freshwater systems. Our study investigated whether mosquito populations of *Culex pipiens*, typically considered sensitive to salt, have adapted due to gradual exposure. Mesocosm experiments were conducted to evaluate responses in life history traits to increasing levels of salinity in three populations along a gradient perpendicular to the North Sea coast. Salt concentrations up to the brackish-marine transition zone (8 g/L chloride) were used, upon which no survival was expected. To determine how this process affects oviposition, a colonization experiment was performed by exposing the coastal population to the same concentrations. While concentrations up to the currently described LD50 (4 g/L) were surprisingly favored during egg laying, even the treatment with the highest salt concentration was incidentally colonized. Differences in development rates among populations were observed, yet the influence of salinity was evident only at 4 g/L and higher, resulting in only a one-day delay. Mortality rates were lower than expected, only reaching 20% for coastal and inland populations and 41% for the intermediate population at the highest salinity. Sex ratios remained unaffected across the tested range. The high tolerance to salinity for all key life-history parameters across populations suggests that *Culex pipiens* is unlikely to shift its distribution in the foreseeable future, with potential implications for the disease risk of associated pathogens.

Keywords: Adaptation; *Culex pipiens*; Environmental change; Mosquito; Population dynamics; Oviposition experiments; Salinization

4

4.1 Introduction

Salinization of fresh water in coastal areas, especially in low-lying deltas, is a natural process that is currently exacerbated by anthropogenic drivers, such as climate change-induced sea level rise, land subsidence and saline ground water seepage, strengthened by the removal of overlying freshwater (van Baaren & Oude Essink, 2009). Saltwater infiltration is commonly acknowledged to negatively affect agricultural yield and freshwater ecosystem services (Bonte & Zwolsman, 2010). The underlying physical processes of salinization are relatively well described (Khan et al., 2011; Lassiter, 2021), and animal diversity at large is understood to decrease under transitory conditions (Telesh et al., 2013). However, little is known about the direct and indirect effects of salinization on animal populations inhabiting (currently freshwater) ecosystems in deltas, especially for species that are disease vectors.

The cosmopolitan house mosquito *Culex pipiens* species complex is a known vector for a variety of pathogens, including West Nile virus, Usutu and avian malaria (Bravo-Barriga et al., 2016; Gutiérrez-López et al., 2016; Hubálek, 2008; Kazlauskienė et al., 2013). It has a wide habitat tolerance, ranging from clean rainwater-filled containers to strongly polluted temporal waterbodies, such as ground puddles, and even manure tanks (Becker et al., 2013; Rejmánková et al., 2013). Similar to other mosquito larvae typically associated with freshwater, it accumulates organic osmolytes to combat ionic pressure instead of active ion transport (Chown & Nicolson, 2004) and is known to be quite vulnerable to changes in salinization relative to other mosquito species (Abou-Attia et al., 2000; Kenawy et al., 2013; Kengne et al., 2019) with a median lethal dose (LD50) of 4 g/L and a lethal dose (LD100) of 6-10 g/L chloride for acute salinity stress (Brown & Platzer, 1978; Chidester, 1916; Kengne et al., 2019).

Although a variety of responses to salinization exist among invertebrates (Chown & Nicolson, 2004), general trends exist in the whole invertebrate community. Salinization has been shown to shape insect community structures, negatively affecting diversity (Bleich et al., 2011; Silberbush et al., 2005) via decreased food availability (Ersoy et al., 2022; van Dijk et al., 2019). Although mosquitoes have previously been described to react quite similarly (Balasubramanian et al., 2019; Telesh et al., 2013), it has also been hypothesized that their short generation time (when compared to that of many other macrofauna species, including their predators (Verberk et al., 2008)) might enable mosquitoes to adapt faster (Carlson et al., 2014; Martin & Palumbi, 1993; Thomas et al., 2010). This could subsequently cause a relative increase in population size in transitory systems due to the alleviation of predation pressure and the relative increase in food resources (Silberbush et al., 2005). Such a fast adaptation rate is observed for a variety of other stressors, such as pesticides (Hamdan et al., 2005; Nazni et al., 2005; Ser & Cetin, 2019). These adaptations are similar to the response to salinization, i.e., by affecting the excretion of harmful compounds (Asakura, 1980; Chown & Nicolson, 2004). This renders it likely that mosquitoes are better able to adapt to increasing salinity than other insect species.

Salinization affects mosquito habitat quality and may thus lower larval survival. However, this depends on how well the larvae are adapted to temporary (i.e., flooding) and continuous salinization events and processes, causing species-specific effects (Kengne et al., 2019). These adaptations in osmoregulation include physiological (reduced surface area of anal papillae or active transport of ions) (Akhter et al., 2017, 2017; Donini et al., 2007) and behavioral adaptations (increased metabolism and uptake of organic compounds in hemolymph) (Aly & Dadd, 1989; Bradley, 1987; Bradley & Phillips, 1976; De Brito Arduino et al., 2015; Donini et al., 2007; Patrick & Bradley, 2000), resulting in tolerance that changes across life stages (Mottram et al., 1994) and differ between sexes (Alcalay et al., 2018). Namely, female mosquitoes tend to be less strongly selected for early maturation, which may lead to prolonged exposure to stress as compared to males (Boerlijst et al., 2023). With time, has adaptations to salinization caused species-specific preferences during oviposition (Boerlijst et al., 2023; Navarro et al., 2003; D. M. Roberts & Irving-Bell, 1997; Silberbush et al., 2014), further shaping mosquito community composition.

At the population level, commonly considered intolerant species such as *Culex pipiens* s.l. (hereafter denoted as *Cx. pipiens*) might be affected by salinization in a variety of ways. Salinization might cause i) no change when tolerance via for instance plastic behavior proves sufficient, ii) local extinction of the species if tolerance is insufficient, iii) displacement when unfavorable conditions are perceived during ovipositing, or iv) local adaptation leading to possibly increased tolerance due to gradual, continuous exposure.

This study aimed to evaluate whether (local) adaptation to salinization occurred, by quantifying and comparing the tolerance of *Cx. pipiens* populations along a gradient from coast to inland. We expected increasing levels of adaptation (i.e. lower mortality, more rapid development and a balanced sex-ratio) closer to the coast as a result of gradual exposure, To this end we performed a mesocosm experiment. We varied concentrations from zero to eight grams of chloride per liter with intervals of two grams, i.e., from freshwater to the predicted maximum inland surface water concentration of 7.5 g/L Cl- (Delsman et al., 2020), or the brackish-marine transition zone (Dahl, 1956), at almost half the concentration of sea water.

4.2 Materials and methods

4.2.1 Collection and rearing of experimental populations

Culex pipiens egg rafts were collected during the two days prior to the start of an experimental round from one set of naturally colonized black plastic mesocosms in peri-urban areas of the cities of Leiden, Utrecht and Nijmegen, representing coastal (7 km to sea), intermediate (43 km to sea) and inland (108 km to sea) mosquito populations, respectively. All populations were collected at similar altitude (2-5 m asl). For this purpose, the mesocosms were filled with 6 liters of hypertrophic water (100 mg N-total), after which they were placed under tree cover. The larvae were subsequently allowed to hatch in 50 mL Falcon tubes, where they were kept at ambient temperature until the start of the experiment. Previous pilot studies have indicated that this type of experiment attracts *Cx. pipiens* and Culiseta annulata only (Boerlijst et al., 2023; Dellar et al., 2022).The collected egg rafts were distinguished from those of Culiseta annulata by their difference in size (Chapman et al., 2020; Sames et al., 2005).

4.2.2 Experimental setup

The setup consisted of 45 white plastic 12 L mesocosms, each with a 200-Watt aquarium heater. The experiments were conducted under standardized outdoor conditions (Boerlijst et al., 2023) at the Hortus botanicus, Leiden, The Netherlands. The aquarium heaters were programmed at a minimum temperature of 20°C for optimal development, whilst allowing for natural fluctuations, so that the development was representative of field conditions during the peak of the Dutch mosquito season (Beck-Johnson et al., 2017; Boerlijst et al., 2023; De Majo et al., 2019). Namely, as increased temperature heightens metabolism, ion uptake and transport may be increased, making it imperative to work under such conditions.

All 45 mesocosms were filled with eight liters of dechlorinated tap water (kept at constant level during the experiments), a natural concentration of microbes, a high concentration of nutrients and a specific concentration of sea salt (Jozo, Rotterdam, The Netherlands). For the natural concentration of microbes, one liter of water from a local lake was filtered per liter of tap water using a 250 μm plankton net and 53 μm collector. The high concentration of nitrogen prevents food from being a limiting factor and thus minimalizes cannibalism (Koenraadt & Takken, 2003). This was achieved by adding 20 mg/L N in the form of dry cow manure (2.4% N, 1.5% P2O5, and 3.1% K2O) to the water. The mesocosms were randomly allocated to five increasing concentrations of commercially available sea salt – 0 g/L, 2 g/L, 4 g/L, 6 g/L, and 8 g/L Cl – and split into two rounds of experiments due to spatial constraints, which are described below. The treatments were representative of freshwater (Oude Essink et al., 2010), the highest measured salinity in a Dutch ditch (Geest et al., 2022), the LD50 (Kengne et al., 2019), the highest measured salinity in seepage water (Geest et al., 2022), and the highest reported LD100 for *Cx. pipiens* (Kengne et al., 2019), respectively (Table 4.1). In the first round, 0 g/L, 2 g/L, and 6 g/L Cl- were used, and in the second round, 0 g/L, 4 g/L, and 8 g/L Cl- were used.

4

Table 4.1 *Conversion table salinity treatments*

	Chloride			*Total salts*		
	g/L (‰)	*ppm*	*%*	*g/L (‰)*	*ppm*	*%*
Fresh water	0.0	0	0.0	0.0	0	0.0
Maximum ditch	2.0	2002	0.2	3.6	3604	0.4
LD50	4.0	4005	0.4	7.3	7308	0.7
Maximum seepage	6.0	6007	0.6	11.0	11013	1.1
LD100	8.0	8009	0.8	14.6	14617	1.5
Typical sea water	18.9	18921	1.9	34.5	34539	3.5

For each of the concentrations, a mixture of water, microbes, nutrients, and sea salt was prepared (Boerlijst et al., 2023; Dellar et al., 2022), and salt was added over the course of four days in equal parts to limit osmotic stress to the microbial community. The mixture was thereafter covered with fine mesh (0.1 mm) to prevent additional colonization and subsequently left to acclimatize for a period of two weeks. After the acclimation period, the water was divided over the experimental mesocosms using a 500 μm sieve to filter out any detritus and macroinvertebrates. After filtering, 100 second instar larvae were added, and the aquarium heaters were turned on. Allocation of the populations and saline concentrations was performed in a Latin square, leading to 5 replicates for each population-concentration combination. During the experiment, the mesocosms were once again closed off using mesh to prevent predation and colonization from the outside and to ensure that the emerged mosquitoes could not escape. Temperature, chlorophyll-a concentration, turbidity, and conductivity were measured as potential covariates using a Hach HQ40d multi and Turner designs Aquafluor. Before the second round of the experiment, the original mixtures were collected, and the concentrations were increased from 2 g/L to 4 g/L and from 6 g/L to 8 g/L. The mixtures were once again left to acclimatize and were subsequently allocated to a new Latin square.

4.2.3 Measurements of population parameters

Larval development was measured five days a week. First, the water was stirred clockwise once with a 400 mm wide Ø 200 μm sieve to create a circular water flow and prevent the larvae from diving. The sieve was subsequently used to collect the larvae by fully submerging the sieve and moving it counterclockwise twice. All the collected larvae were morphologically characterized to developmental stage by using the size of the head capsule as a morphological indicator (Becker et al., 2010). The identifications were compared daily with a previously reared reference collection of *Cx. pipiens* developmental stages. The procedure was repeated up to five times until at least twenty larvae were sampled.

Pupa were collected daily, after which they were allowed to emerge in 50 ml falcon tubes. Sex was determined based on characteristics, including plumose/pilose antennae and the length of the palps (Becker et al., 2010). The proportion of total survival was determined by dividing the number of emerged adults by the original density of 100 larvae. The proportion of survival , used for visualization, were calculated by subtracting the mean of the control per population from the absolute survival rate. The time to pupation was determined after completion of the experiment. Time to pupation was defined as the interval between the start of the experiment and the first day upon which at least 50% of the subsampled larvae had turned/developed into pupae. The median time to emergence was determined by calculating the interval between the start of the experiment and capture of 50% of the emerged adults. When no more pupae and adult mosquitoes were found for two subsequent days in a mesocosm, it was assumed that there were no living mosquitoes left and the mesocosm was closed off.

4.2.4 Ovipositioning behavior

The ovipositioning behavior of the coastal population was determined in a separate experiment at the Hortus botanicus Leiden, The Netherlands. Five clusters – each consisting of one black, plastic 8 L bucket for each of the five salt concentrations – were placed around the botanical gardens at a distance of at least 58 m from each other to prevent the clusters from interfering with each other. The water, microbial community and salinity levels were prepared as described in the previous section. Ovipositioning behavior was recorded by daily counts of egg rafts per mesocosm for a total of twelve days. Encountered egg rafts were removed to minimize the positive feedback caused by their presence (Bruno & Laurence, 1979).

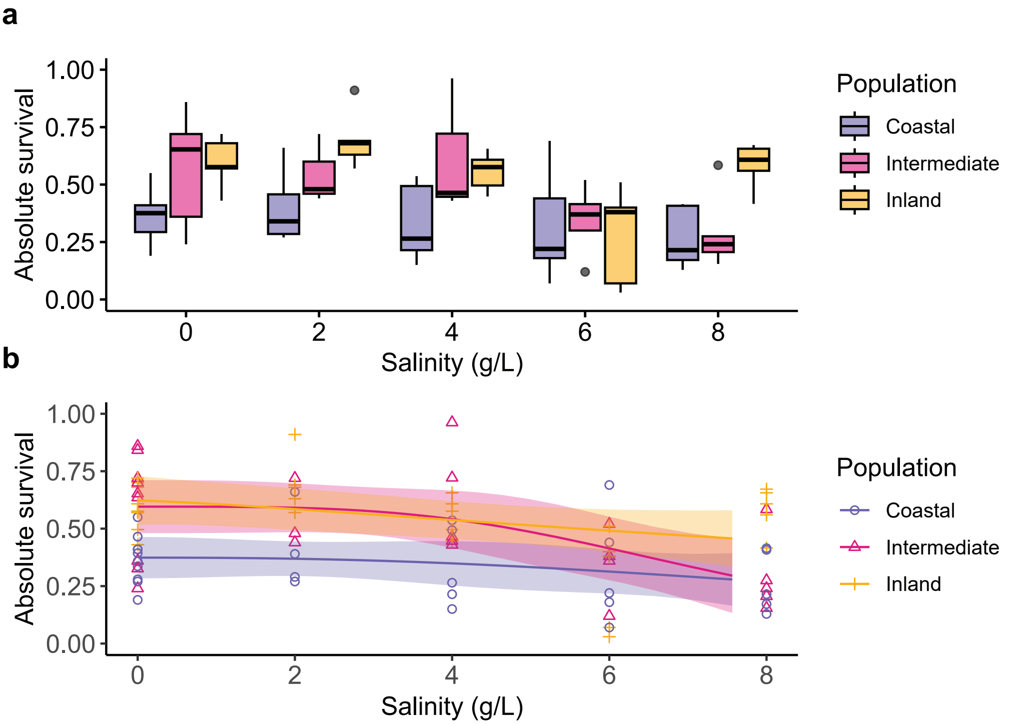


Figure 4.1 *Proportion of normalized total survival per population across increasing salinization levels as a. boxplot with outliers as dots and b. dose-response curve with standard error. Total survival is depicted as the number of emerged adults at the end of the experiment as a fraction of the initial number of larvae.*

4.2.5 Statistical analyses

All data were analyzed in R version 4.2.2 (R Core Team, 2018). Variance across experimental rounds was normalized based on the observed variance across the experimental rounds per population per salinity. Log-logistic regression was used to determine the LD50 and LD100 using the drc package (Ritz et al., 2015). Linear mixed effects models were used to test for (normalized) differences in survival, development time (to pupation and emergence) and sex ratio across the different salinity levels. The salinity level, population, experimental round, average turbidity, conductivity and chlorophyll-a concentration were included as covariates. The individual mesocosms were included as random effect. The effect on ovipositioning behavior was explored similarly; a linear mixed model was applied using salinity level as main effects and day and location as random variables. All models (Supplementary Table 4.1) were optimized by Akaike information criterion using stepwise regression with backwards elimination. Dependent variables were tested for normality and assessed using quantile quantile plots and Levene's test ($P = 0.05$).

4.3 Results

4.3.1 Effect of salinity on total proportion of survival

The total proportion of survival decreased with increasing salinity for all populations ($F_{(4,85)}=5.60$, $p<0.001$, partial $\eta 2 = 0.281$), with 18%, 42% and 20% ($p<0.001$, $p=0.005$, and $p=0.001$ for coastal, intermediate and inland respectively; Figure 4.1) from 4 g/L onward (Supplementary Table S4.2). Differences in slope were detected between the coastal and intermediate population ($t_{(30,27)} = -2.51$, p.adj<0.001), coastal and inland population (($t_{(30,28)} = -3.83$, adj =0.031), but not between the intermediate and inland populations ($t_{(28,27)}=0.69$, p. adj >0.05).

4.3.2 Effect of salinity on development rates

A minor increase in the time to pupation (Supplementary Figure S4.1) and time to emergence (Figure 4.2) was detected with increasing salinity. Development to emergence was equally slowed for all populations. On average, the larvae exposed to 8 g/L took 1 day longer to emerge than those exposed to 0 g/L NaCl ($t(4,71) = -2.849$, $p<0.041$, partial $\eta 2 = 0.412$; Figure 4.2; Supplementary Table S4.3).

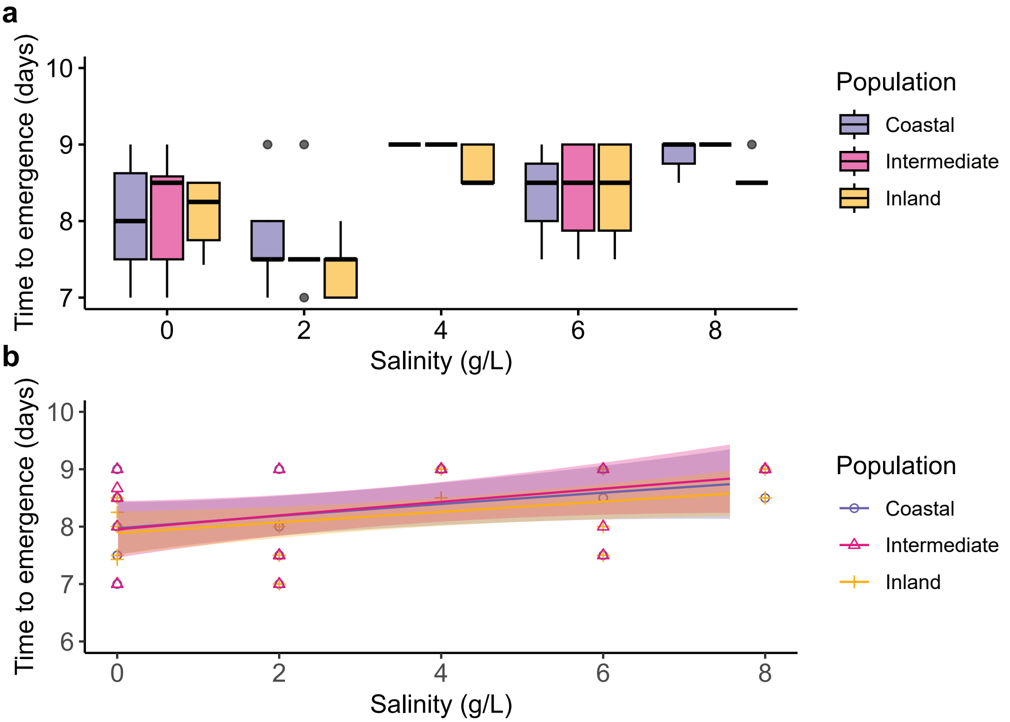


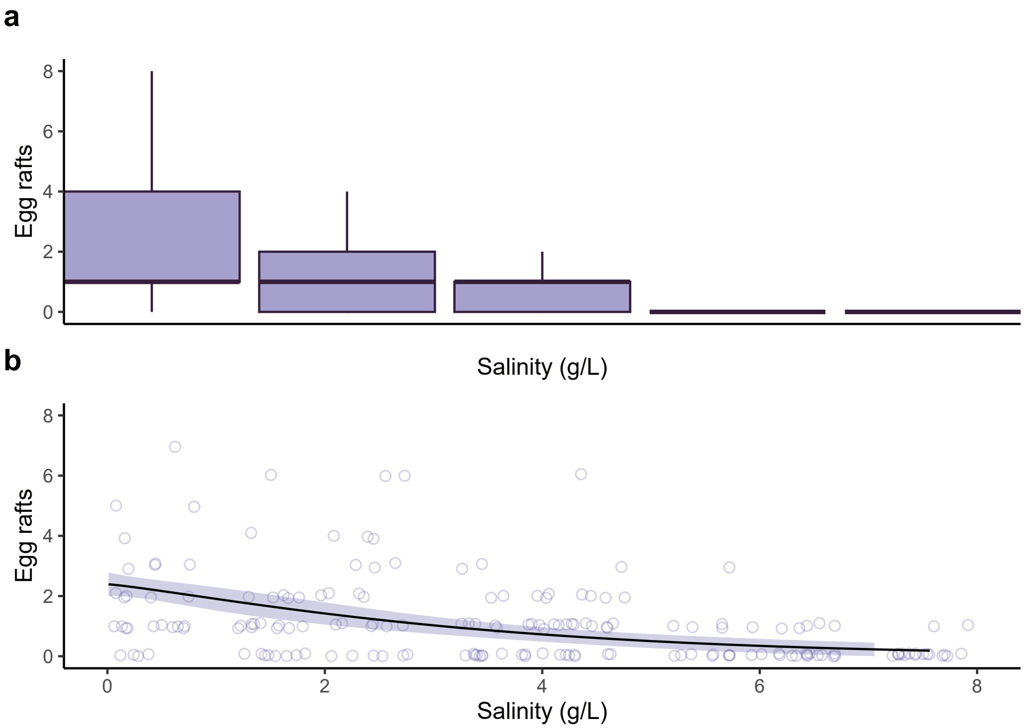
Figure 4.2 *Normalized median time to emergence in days per population across increasing salinization levels as a. boxplot with outliers as dots and b. dose-response curve with standard error.*

4.3.3 Effect of salinity on sex ratio

A minor difference in sex ratio was detected with increasing salinity or among any of the populations ($F(2,62) = 3.266$, $p=0.045$, partial $\eta 2 = 0.102$; Figure 4.3; Supplementary Table S4.4), between.the coastal and inland populations (p.adj=0.013).

4.3.4 Effect of salinity on ovipositioning behavior

Oviposition decreased with increasing salt concentration ($F(4,297) = 25.863$, $p<0.001$, partial $\eta 2 =0.273$; Figure 4.3; Table 4.2; Supplementary Table S4.5). The average oviposition rate decreased by 67% to 1.5 rafts or approximately 300 eggs (Becker et al., 2010) at 2 g/L and subsequently by 11% to 1 or approximately 200 eggs at 4 g/L. Oviposition rates at 6 g/L were almost negligible at 9%.



4

Figure 4.3 *Daily ovipositioning behavior across increasing salinization levels, showing the number of egg rafts for each salinization level as a. boxplot and b. dose-response curve with standard error.*

Table 4.2 *Summary statistics on the ovipositioning rates for each salinity comparison*

Contrast	Estimate	SE	t ratio	Adj. p value
0 g/L - 2 g/L	1.52	0.665	2.285	0.1997
0 g/L - 4 g/L	1.92	0.667	2.879	0.07
0 g/L - 6 g/L	4.69	0.665	7.058	<.0001***
0 g/L - 8 g/L	5.79	0.665	8.711	<.0001***
2 g/L - 4 g/L	0.4	0.663	0.604	0.9724
2 g/L - 6 g/L	3.17	0.661	4.802	0.0016**
2 g/L - 8 g/L	4.27	0.661	6.464	0.0001***
4 g/L - 6 g/L	2.77	0.663	4.183	0.0055**
4 g/L - 8 g/L	3.87	0.663	5.841	0.0002***
6 g/L - 8 g/L	1.1	0.661	1.663	0.4824

4.4 Discussion and conclusion

Contrary to our expectations, our results suggest that investigated populations of Cx *pipiens* are highly tolerant to salinization, irrespective of their proximity to the current coastline. At the highest salinity (Figure 4.1), representative of almost half the concentration of sea water, more than half of the larvae survived for all tested populations, instead of the expected 0% (Brown & Platzer, 1978; Chidester, 1916; Kengne et al., 2019). Differences in development rates among populations were observed, yet the influence of salinity was evident only at 4 g/L or higher, resulting in a minor delay (Figure 4.2). The sex ratios remained unaffected across the tested range, indicating no expected effect on potential population growth (Figure 4.3). Our data additionally suggest that, although concentrations up to the previously described LD50 (4 g/L) were favored during egg laying, *Cx. pipiens* readily lays eggs under conditions of up to 6 g/L Cl- and, incidentally, under 8 g/L Cl-. This finding is in line with observational data, as *Cx. pipiens* has recently been repeatedly observed to inhabit Dutch salt marches (pers. comm. J.G. van der Beek), which suggests a more congruent link between ovipositioning behavior and larval survival than has been described for other species (D. Roberts, 1996; D. M. Roberts & Irving-Bell, 1997; Yee et al., 2020).

Our observations are striking in contrast to the previously described LD100 of 6-7 g/L Cl- in the USA and France (Brown & Platzer, 1978; Chidester, 1916; Kengne et al., 2019). There are several methodological differences that exist between the current study and previous literature: i) the use of second-instar larvae, which might increase the potential for physiological changes in response to saline conditions (Bradley, 1987) compared to the use of older larvae; ii) the use of eutrophic conditions, which, by increasing the energy budget of the larvae, might allow for higher metabolic rates, increasing the ability to expel the ionic waste (Bradley & Phillips, 1976); and, finally, iii) gradual acclimation of the locally sourced microbial community, which might have allowed for a higher microbial abundance and thus food availability during the experiment. The latter might have allowed for increased uptake of organic compounds, which may reduce the effects of the water's osmolality (De Brito Arduino et al., 2015). While the relevance of each of these differences in setup cannot be distinguished with the current setup, the difference in total survival between our study and the earlier findings is far greater than might be explained by changes in methodology.

As our experimental setting is more representative of field conditions, the currently described responses might be more ecologically relevant than those described in previous studies under controlled conditions in the laboratory, as these generally use alternate food sources (e.g. fish feed), tap water without a natural microbial community (Kauffman et al., 2017), or laboratory-reared communities of a laboratory colony with a single subspecies. Given the ecological relevance of the setup applied, the observed pattern might be representative of populations in the Netherlands and possibly even for many other, low-lying deltas. Based on these results, we speculate that similar patterns may exist for other mosquito species that inhabit lowland delta areas, such as Culiseta morsitans, *Culex* modestus and perhaps even Aedes aegypti, which would imply that the current LD50 and LD100 should be reassessed. Taken together, the difference in the responses of our study and laboratory studies suggests that, while a wide range of mosquito species are typically associated withfreshwater systems (Multini et al., 2021), they may exhibit substantial plasticity and/or (local) adaptation to increasing salinization.

The current results suggest that coastal house mosquito populations will persist and will not show salinity-induced inland dispersal or local reductions in survival. The ecological implications are that they may instead locally increase in population size, despite the presence of predators. Many freshwater predator groups, including dragonflies and damselflies (Golovatyuk & Shitikov, 2016) and mayflies and true bugs (Dunlop et al., 2008), have longer generation times and may be vulnerable to salinization within the range tested. However, this assumption remains to be tested. Species diversity in transitory systems tends to decrease between freshwater and saline water (Bleich et al., 2011; Telesh et al., 2013), while total insect abundance may remain unchanged (Silberbush et al., 2005). Consequently, species that are able to persist in such systems may experience alleviation of predation pressure, causing population sizes to increase over time and strengthening nuisance and disease risk. However, additional information is needed, as many studies on the tolerances of predator species are prone to methodological limitations similar to those of prior work on mosquitoes themselves. Nevertheless, house mosquito nuisance in coastal areas is likely to persist during the foreseeable future, and our results suggest that it is not unlikely that other mosquito species in coastal areas are similarly able to adapt to increasing salt levels even though their predators cannot.

Acknowledgements

Gertjan Geerling is gratefully acknowledged for his help in the collection of egg rafts. We thank Hortus botanicus Leiden for allowing us to conduct our experiments on their premises. We thank Toos van Peuzelen for their helpful discussions and support during the conceptualization and collection of the data.

Author contributions

SB and MS conceived the general idea for the experiments. SB set up the experiments, and AG and LA carried out the measurements. Interpretation was performed by SB together with EB, RB, PB and MS. SB carried out all statistical analyses, together with PB and MS. All the authors contributed critically to the drafts and gave final approval for publication.

Availability of data and materials

Data supporting the conclusions of this article are included within the article and its additional files. The original datasets used and analyzed during the present study are freely and openly available within the supplementary information files.

Ethics approval and consent to participate

Not applicable. Ethical clearance was not needed for this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Electronic appendix

Time to pupation

Lambda = 2
Formula: ((Day^lambda - 1)/lambda) ~ Treatment + (1 | Cosm)
Data: DR_MTP

Analysis of Variance Table

	npar	Sum Sq	Mean Sq	F value
Treatment	4	2006.8	501.7	7.2123

REML criterion at convergence: 1608

Scaled residuals:

Min	1Q	Median	3Q	Max
-2.4045	-0.4825	0.2376	0.6180	2.1521

Random effects:

Groups	Name	Variance	Std.Dev.
Cosm	(Intercept)	0.00	0.00
Residual		72.94	8.54

Number of obs: 228, groups: Cosm, 45

Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	28.0353	0.9263	30.265
Treatment2	-6.4149	1.4862	-4.316
Treatment4	7.4111	1.8609	3.983
Treatment6	1.4353	1.7330	0.828
Treatment8	6.6869	1.8866	3.544

Correlation of Fixed Effects:

	(Intr)	Trtmn2	Trtmn4	Trtmn6
Treatment2 -	0.623			
Treatment4 -	0.498	0.310		
Treatment6	-0.535	0.333	0.266	
Treatment8	-0.491	0.306	0.244	0.262

optimizer (nloptwrap) convergence code: 0 (OK)

(Intercept)	Treatment2	Treatment4	Treatment6	Treatment8
1.479537e-80	2.393689e-05	9.253775e-05	4.084389e-01	4.802100e-04

Contrast	estimate	SE	df	t.ratio	p.value
Treatment0 - Treatment2	6.415	1.49	48.5	4.299	0.0008
Treatment0 - Treatment4	-7.411	1.86	114.8	-3.977	0.0011
Treatment0 - Treatment6	-1.435	1.74	83.3	-0.825	0.9221
Treatment0 - Treatment8	-6.687	1.89	118.0	-3.540	0.0051
Treatment2 - Treatment4	-13.826	1.99	200.8	-6.936	<.0001
Treatment2 - Treatment6	-7.850	1.88	101.5	-4.183	0.0006
Treatment2 - Treatment8	-13.102	2.02	134.3	-6.497	<.0001
Treatment4 - Treatment6	5.976	2.18	159.6	2.737	0.0530
Treatment4 - Treatment8	0.724	2.30	183.5	0.314	0.9979
Treatment6 - Treatment8	-5.252	2.20	194.7	-2.382	0.1245

Note: contrasts are still on the (scale
Degrees-of-freedom method: kenward-roger
P value adjustment: tukey method for comparing a family of 5 estimates

Time to emergence
Lambda = 2
Formula: ((Day^lambda - 1)/lambda) ~ Treatment + (1 | Cosm)
REML criterion at convergence: 1256

Analysis of Variance Table

	npar	Sum Sq	Mean Sq	F value
Treatment	4	2006.8	501.7	7.2123

Scaled residuals:

Min	1Q	Median	3Q	Max
-2.24368	-0.80490	0.09433	0.95199	1.45162

Random effects:

Groups	Name	Variance	Std.Dev.
Cosm	(Intercept)	0.00	0.00
Residual		69.56	8.34

Number of obs: 180, groups: Cosm, 44
Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	30.713	1.011	30.366
Treatment2	-2.820	1.505	-1.874
Treatment4	7.587	2.379	3.189
Treatment6	1.347	1.951	0.690
Treatment8	6.631	2.317	2.861

Correlation of Fixed Effects:

	(Intr)	Trtmn2	Trtmn4	Trtmn6
Treatment2	-0.672			
Treatment4	-0.425	0.286		
Treatment6	-0.518	0.348	0.220	
Treatment8	-0.436	0.293	0.186	0.226

optimizer (nloptwrap) convergence code: 0 (OK)

(Intercept)	Treatment2	Treatment4	Treatment6	Treatment8
3.139731e-71	6.262380e-02	1.695946e-03	4.908814e-01	4.742289e-03

Contrast	estimate	SE	df	t.ratio	p.value
Treatment0 - Treatment2	2.820	1.51	37.4	1.864	0.3541
Treatment0 - Treatment4	-7.587	2.39	129.2	-3.172	0.0160
Treatment0 - Treatment6	-1.347	1.97	60.0	-0.685	0.9591
Treatment0 - Treatment8	-6.631	2.33	116.2	-2.849	0.0407
Treatment2 - Treatment4	-10.407	2.44	160.6	-4.273	0.0003
Treatment2 - Treatment6	-4.167	2.02	66.9	-2.062	0.2486
Treatment2 - Treatment8	-9.451	2.37	122.5	-3.983	0.0011
Treatment4 - Treatment6	6.240	2.74	133.5	2.277	0.1590
Treatment4 - Treatment8	0.956	3.01	160.6	0.318	0.9978
Treatment6 - Treatment8	-5.284	2.69	174.0	-1.968	0.2862

Note: contrasts are still on the (scale
Degrees-of-freedom method: kenward-roger
P-value adjustment: tukey method for comparing a family of 5 estimates

Sex-ratio

Lambda = 0.3838384
Formula: ((SRlog_corrected_rel^lambda - 1)/lambda) ~ City + (1 | Cosm)
REML criterion at convergence: 151

Analysis of Variance Table

	npar	Sum Sq	Mean Sq	F value
City	2	0.23147	0.11573	3.0896

Scaled residuals:

Min	1Q	Median	3Q	Max
-1.43023	-0.85746	0.08101	0.65570	2.71416

Random effects:

Groups	Name	Variance	Std.Dev.
Cosm	(Intercept)	0.0000	0.0000
Residual		0.6493	0.8058

Number of obs: 62, groups: Cosm, 45

Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	-1.9143	0.1758	-10.887
Intermediate	0.1141	0.2487	0.459
Inland	0.4615	0.2518	1.833

Correlation of Fixed Effects:

	(Intr)	CtyUtr
Intermediate	-0.707	
Inland	-0.698	0.494

optimizer (nloptwrap) convergence code: 0 (OK)

(Intercept)	Intermediate	Inland
1.520655e-15	6.481710e-01	7.199709e-02

4

Total proportion of survival

Lambda = 0.5858586
Formula: ((ASR_corrected_rel^lambda - 1)/lambda) ~ City + Treatment + (1 | Cosm)
REML criterion at convergence: 36.4

	npar	Sum Sq	Mean Sq	F value
City	2	0.63438	0.31719	8.2936
Treatment	4	0.85740	0.21435	5.6047

Scaled residuals:

Min	1Q	Median	3Q	Max
-2.06181	-0.55392	-0.00123	0.56390	2.81515

Random effects:

Groups	Name	Variance	Std.Dev.
Cosm	(Intercept)	0.01721	0.1312
Residual		0.05761	0.2400

Number of obs: 85, groups: Cosm, 45

Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	-0.66000	0.06881	-9.591
Intermediate	0.20807	0.07248	2.871
Inland	0.23742	0.06967	3.408
Treatment2	0.14136	0.09428	1.499
Treatment4	-0.05414	0.09205	-0.588
Treatment6	-0.33562	0.09216	-3.642
Treatment8	-0.22498	0.09021	-2.494

Correlation of Fixed Effects:

	(Intr)	Intermediate	Inland	Trtmn2	Trtmn4	Trtmn6
Intermediate	-0.510					
Inland	-0.485	0.481				
Treatment2	-0.467	-0.007	-0.051			
Treatment4	-0.497	-0.003	0.005	0.493		
Treatment6	-0.499	0.027	-0.022	0.364	0.371	
Treatment8	-0.501	-0.002	-0.016	0.371	0.379	0.517

(Intercept) Intermediate Inland Treatment2 Treatment4 Treatment6
Treatment8
9.914e-15 5.302e-03 1.050e-03 1.379e-01 5.582e-01 4.919e-04 1.480e-
02

Contrast	estimate	SE	df	t.ratio	p.value
Coastal - Intermediate	-0.2081	0.0744	78.0	-2.798	0.0176
Coastal - Inland	-0.2374	0.0713	69.7	-3.332	0.0039
Intermediate - Inland	-0.0294	0.0742	77.5	-0.395	0.9175

Results are averaged over the levels of: Treatment
Note: contrasts are still on the (scale
Degrees-of-freedom method: kenward-roger
P value adjustment: tukey method for comparing a family of 3 estimates

Contrast	estimate	SE	df	t.ratio	p.value
Treatment0 - Treatment2	-0.1414	0.0945	63.9	-1.496	0.5691
Treatment0 - Treatment4	0.0541	0.0922	62.4	0.587	0.9765
Treatment0 - Treatment6	0.3356	0.0923	62.3	3.637	0.0049
Treatment0 - Treatment8	0.2250	0.0902	60.8	2.493	0.1056
Treatment2 - Treatment4	0.1955	0.0942	41.6	2.076	0.2494
Treatment2 - Treatment6	0.4770	0.1054	75.3	4.526	0.0002
Treatment2 - Treatment8	0.3663	0.1037	74.9	3.533	0.0062
Treatment4 - Treatment6	0.2815	0.1035	74.9	2.719	0.0605
Treatment4 - Treatment8	0.1708	0.1017	74.5	1.680	0.4521
Treatment6 - Treatment8	-0.1106	0.0897	38.5	-1.233	0.7323

Results are averaged over the levels of: City
Note: contrasts are still on the (scale
Degrees-of-freedom method: kenward-roger
P value adjustment: tukey method for comparing a family of 5 estimates

Slope

group1	group2	n1	n2	statistic	df	p	p.adj
Coastal	Inland	30	28	-3.8344723	55.9619	0.000321	0.000963
Coastal	Intermed.	30	27	-2.5086395	49.80637	0.015	0.031
Inland.	Intermed.	28	27	0.6902263	47.41384	0.493	0.493

4

4

Lambda = -0.1818182
Formula: ((Egg_rafts^lambda - 1)/lambda) ~ Treatment + (1 | Location) + (1 | Day) + (1 | Cosm)
Random effects: REML criterion at convergence: 1504.2

Analysis of Variance Table

	npar	Sum Sq	Mean Sq	F value
Treatment	4	887.19	221.8	25.863

Scaled residuals:

Min	1Q	Median	3Q	Max
-2.23494	-0.70171	0.00891	0.77327	2.20855

Random effects:

Groups	Name	Variance	Std.Dev.
Cosm	(Intercept)	0.3772	0.6142
Day	(Intercept)	0.8563	0.9254
Location	(Intercept)	0.6625	0.8139
Residual		8.5758	2.9284

Number of obs: 297, groups: Cosm, 25; Day, 12; Location, 5

Fixed effects:

	Estimate	Std. Error	t value
(Intercept)	-1.1741	0.6538	-1.796
Treatment2	-1.5191	0.6648	-2.285
Treatment4	-1.9195	0.6666	-2.880
Treatment6	-4.6923	0.6648	-7.059
Treatment8	-5.7911	0.6648	-8.711

Correlation of Fixed Effects:

	(Intr)	Trtmn2	Trtmn4	Trtmn6
Treatment2	-0.514			
Treatment4	-0.513	0.504		
Treatment6	-0.514	0.506	0.504	
Treatment8	-0.514	0.506	0.504	0.506

(Intercept)	Treatment2	Treatment4	Treatment6	Treatment8
7.357064e-02	2.303516e-02	4.280169e-03	1.260673e-11	2.397374e-16

Contrast	estimate	SE	df	t.ratio	p.value
Treatment0 - Treatment2	1.52	0.665	16.1	2.285	0.1997
Treatment0 - Treatment4	1.92	0.667	16.3	2.879	0.0700
Treatment0 - Treatment6	4.69	0.665	16.1	7.058	<.0001
Treatment0 - Treatment8	5.79	0.665	16.1	8.711	<.0001
Treatment2 - Treatment4	0.40	0.663	16.0	0.604	0.9724
Treatment2 - Treatment6	3.17	0.661	15.8	4.802	0.0016
Treatment2 - Treatment8	4.27	0.661	15.8	6.464	0.0001
Treatment4 - Treatment6	2.77	0.663	16.0	4.183	0.0055
Treatment4 - Treatment8	3.87	0.663	16.0	5.841	0.0002
Treatment6 - Treatment8	1.10	0.661	15.8	1.663	0.4824

Note: contrasts are still on the (scale
Degrees-of-freedom method: kenward-roger
P value adjustment: tukey method for comparing a family of 5 estimates

Table S4.1 Differences in survival rate over the salinity gradient

Contrast	Estimate	SE	Df	T.ratio	p value
Coastal - intermediate	-0.2081	0.0744	78.0	-2.798	0.0176
Coastal - inland	-0.2374	0.0713	69.7	-3.332	0.0039
Intermediate - inland	-0.0294	0.0742	77.5	-0.395	0.9175

Table S4.2 Summary statistics on the survival ratios for each salinity comparison per population

Population Coastal

Contrast			Estimate	SE	Df	T.ratio	P.value
0 g/L	-	2 g/L	-0.131	0.095	62.9	-1.378	0.6437
0 g/L	-	4 g/L	0.0714	0.0951	63.2	0.751	0.9434
0 g/L	-	6 g/L	0.3519	0.0927	61.5	3.796	0.003
0 g/L	-	8 g/L	0.2125	0.0927	61.7	2.293	0.1611
2 g/L	-	4 g/L	0.2023	0.0998	41.2	2.028	0.2711
2 g/L	-	6 g/L	0.4828	0.1061	73.3	4.551	0.0002
2 g/L	-	8 g/L	0.3434	0.1061	73.3	3.237	0.0152
4 g/L	-	6 g/L	0.2805	0.1064	73.3	2.635	0.0744
4 g/L	-	8 g/L	0.1411	0.1064	73.5	1.327	0.6757
6 g/L	-	8 g/L	-0.1394	0.0946	37.4	-1.473	0.5857

Population Intermediate

Contrast			Estimate	SE	Df	T.ratio	P.value
0 g/L	-	2 g/L	-0.131	0.095	62.9	-1.378	0.6437
0 g/L	-	4 g/L	0.0714	0.0951	63.2	0.751	0.9434
0 g/L	-	6 g/L	0.3519	0.0927	61.5	3.796	0.003
0 g/L	-	8 g/L	0.2125	0.0927	61.7	2.293	0.1611
2 g/L	-	4 g/L	0.2023	0.0998	41.2	2.028	0.2711
2 g/L	-	6 g/L	0.4828	0.1061	73.3	4.551	0.0002
2 g/L	-	8 g/L	0.3434	0.1061	73.3	3.237	0.0152
4 g/L	-	6 g/L	0.2805	0.1064	73.3	2.635	0.0744
4 g/L	-	8 g/L	0.1411	0.1064	73.5	1.327	0.6757
6 g/L	-	8 g/L	-0.1394	0.0946	37.4	-1.473	0.5857

Population Inland

Contrast			Estimate	SE	Df	T.ratio	P.value
0 g/L	-	2 g/L	-0.131	0.095	62.9	-1.378	0.6437
0 g/L	-	4 g/L	0.0714	0.0951	63.2	0.751	0.9434
0 g/L	-	6 g/L	0.3519	0.0927	61.5	3.796	0.003
0 g/L	-	8 g/L	0.2125	0.0927	61.7	2.293	0.1611
2 g/L	-	4 g/L	0.2023	0.0998	41.2	2.028	0.2711
2 g/L	-	6 g/L	0.4828	0.1061	73.3	4.551	0.0002
2 g/L	-	8 g/L	0.3434	0.1061	73.3	3.237	0.0152
4 g/L	-	6 g/L	0.2805	0.1064	73.3	2.635	0.0744
4 g/L	-	8 g/L	0.1411	0.1064	73.5	1.327	0.6757
6 g/L	-	8 g/L	-0.1394	0.0946	37.4	-1.473	0.5857

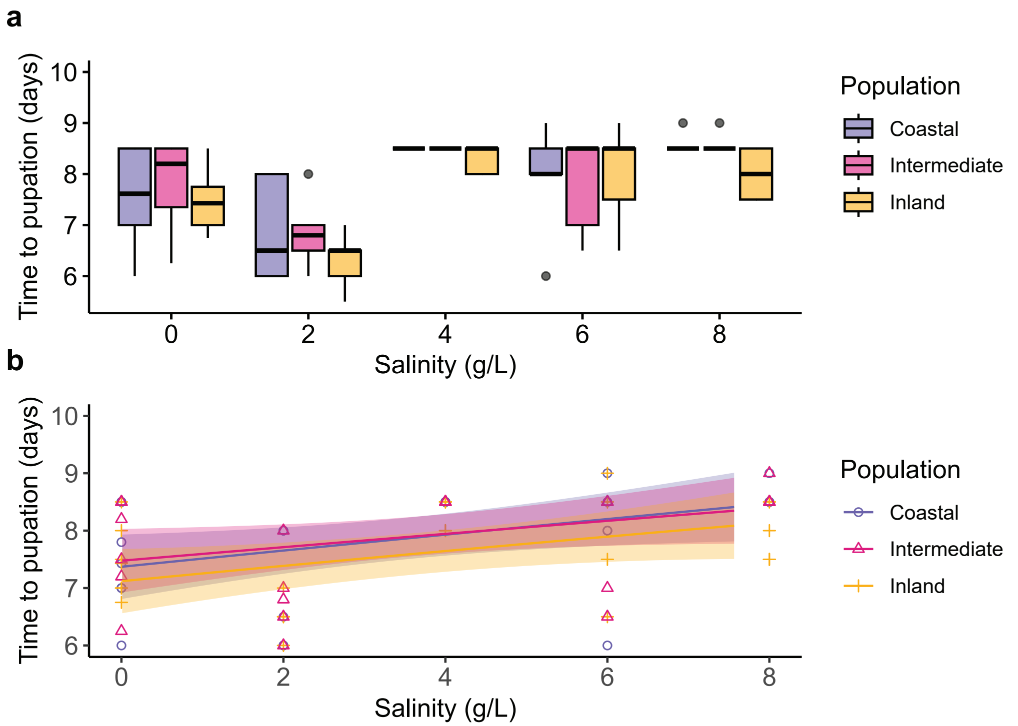


Figure S4.1 *Normalized median time to pupation in days per population across increasing salinization levels as a. boxplot with outliers as dots and b. dose-response curve with standard error.*

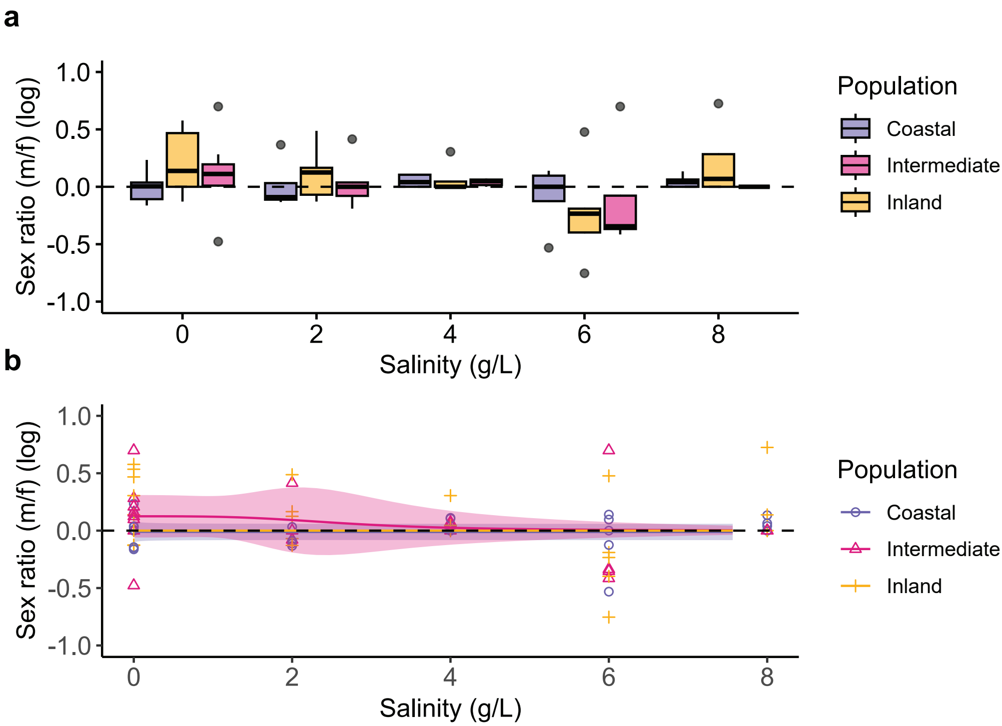


Figure S4.2 *Normalized male to female sex ratio (transformed as natural logarithm) at the end of the experiment per population across increasing salinization levels as a. boxplot with outliers as dots and b. dose-response curve with standard error*

Table S4.3 Model summary statistics on the time to emergence for each salinity comparison and population.

	Estimate	St.E.	t value	p value
(Intercept)	30.713	1.011	30.366	3.139731e-71
Treatment2	-2.82	1.505	-1.874	6.262380e-02
Treatment4	7.587	2.379	3.189	1.695946e-03
Treatment6	1.347	1.951	0.69	4.908814e-01
Treatment8	6.631	2.317	2.861	4.742289e-03

Table S4.4 Model summary statistics on the male:female sex ratio for each population

	Estimate	Std. Error	t value	p value
(Intercept)	-1.9143	0.1758	-10.887	1.52E-15
Inland population	0.1141	0.2487	0.459	6.48E-01
Intermediate population	0.4615	0.2518	1.833	7.20E-02

Table S4.5 Model summary statistics on the ovipositioning behavior for each population

	Estimate	Std. Error	t value	p value
(Intercept)	-1.1741	0.6538	-1.796	7.36E-02
Treatment2	-1.5191	0.6648	-2.285	2.30E-02
Treatment4	-1.9195	0.6666	-2.88	4.28E-03
Treatment6	-4.6923	0.6648	-7.059	1.26E-11
Treatment8	-5.7911	0.6648	-8.711	2.40E-16