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DISEASE ECOLOGY

Tolerance of disease-vector mosquitoes to brackish water and their osmoregulatory ability

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Abstract. Salinity tolerance is an important trait that governs the ecology of disease-vector mosquitoes by determining their choice of larval habitat, and consequently their ecological and geographical distribution. Here, we used laboratory strains to determine the osmotic responses of larvae of obligate freshwater disease-vector mosquitoes (*Aedes aegypti*, *Aedes albopictus*, *Anopheles coluzzii*, *An. gambiae*, *Culex pipiens*, and *Cx. quinquefasciatus*) and assessed their relationship with salinity tolerance. First, we analyzed the acute dose–mortality response of fourth-instar larvae to salinity; then, we measured their hemolymph osmolality after 24-h exposure to varying salinities. We found that *Ae. albopictus* was the most tolerant species, followed by *An. coluzzii*, *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. gambiae*, in decreasing order. *Cx. pipiens* was the least tolerant species. All mosquitoes were hyper-iso-osmoregulators, but with species-specific differences. Specifically, hemolymph osmolality in deionized water varied among species, and *Cx. pipiens* and the two *Aedes* species showed the lowest and highest osmolality. Although all species were osmoconformers at higher salinity values, hemolymph osmolality approached environmental osmolality more rapidly in species of the *Culex* genus, compared with *Aedes* species where it increased slowly. Moreover, hemolymph osmolality in deionized water was significantly correlated with tolerance to salinity across species. This could allow predicting the salinity tolerance of untested species on the basis of their osmoregulatory ability. However, this correlation disappeared when considering the hemolymph osmolality of larvae exposed to salinities higher than deionized water.

Key words: adaptation; *Aedes; Anopheles; Culex;* hemolymph osmolality; lethal concentration; mosquitoes; osmoregulation; salinity tolerance.

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Introduction

Aquatic organisms are often confronted in their habitat with changing salinity to which they have to adapt. According to their species, they display different abilities to regulate their internal milieu (Barton-Browne 1964, Beyenbach and Piermarini 2009, Evans and Claiborne 2009). Therefore, fluctuating aquatic environments are a major source of physiological stress with a more or less pronounced effect, depending on the osmotic gradient intensity and the organism adaptability. Water salinity and ionic composition are major natural abiotic stressors in water habitats and are one of the main environmental constraints that characterize the basic ecological

niche of aquatic organisms (Ward 1992). Accordingly, the ability of aquatic organisms to tolerate varying salinity levels and to adapt to such variations governs several biological processes, and ultimately their eco-geographical distribution (Galat et al. 1988, Williams et al. 1990, Cervetto et al. 1999, Herbst 2001, Willmer et al. 2009).

To survive in aquatic environments where salinity and thus osmotic pressure vary, aquatic arthropods have developed two main physiological strategies: osmoconformation and osmoregulation (Bradley 1987, Charmantier et al. 2009). The vast majority of marine invertebrates are osmoconformers that maintain, for a limited range of fluctuating salinities, their internal osmotic pressure close to the osmotic pressure of the external medium through intracellular and/or extracellular organic osmolytes (Bradley 1987, Torres et al. 2011, Pallarés et al. 2015). Conversely, osmoregulators regulate their hemolymph osmolality mainly by active transport of ions via specialized organs (Bradley 1987, 2008, 2009, Beyenbach and Piermarini 2009, Evans and Claiborne 2009). Although osmoregulatory mechanisms have been extensively studied in marine organisms, knowledge of these mechanisms in freshwater organisms is more limited compared with marine and brackish species. Particularly, the relationship between osmoregulation and salinity tolerance is not well known in freshwater species, despite the fact that salinity tolerance is an important determinant of local adaptation and evolutionary history (Munoz et al. 2008, Pinceel et al. 2013, Arribas et al. 2014). Wigglesworth carried out the first pioneering study on osmoregulation in *Aedes aegypti* and *Culex pipiens* larvae (Wigglesworth 1938). Mosquito larvae, like many other aquatic arthropod larvae, survive in challenging osmotic gradients by regulating the excretion and/or absorption of the available ions in their aquatic habitats. This process involves several morphological and physiological adaptations, reviewed in Beyenbach and Piermarini (2009) (White et al. 2013). Some mosquitoes of the *Culex* and *Culiseta* genera increase their internal osmolality by accumulating organic compounds, such as amino acids (e.g., proline) and sugars (e.g., trehalose), in the hemolymph. This allows them to maintain a stable osmotic gradient with the external environment (Bradley 1987, 1994, Garrett and Bradley 1987, Patrick and Bradley 2000). In other salt-tolerant *Culicidae* species, osmoregulation involves morphological changes in the larval posterior and anterior rectum, and a change in the composition and location of dorsal anterior rectum (DAR) and non-DAR cells that regulate ion excretion and absorption (Bradley 1987, Smith et al. 2008, Beyenbach and Piermarini 2009, Xiang et al. 2012, White et al. 2013). In some species, such as *Aedes taeniorhynchus*, the progressive exposure of mosquito larvae to increasing salinities has selected populations with different levels of adaptation to local conditions, enabling some populations to tolerate salinities in excess of seawater (Bradley and Philips 1977).

Salinity tolerance is an important trait that governs the ecology of disease-vector mosquitoes by determining the choice of larval habitat, and consequently their ecological and geographical distribution, and ultimately, the disease transmission epidemiology (Ramasamy and Surendran 2011, Ramasamy et al. 2011, Yee et al. 2014, Surendran et al. 2018). Mosquito larvae are considered to be mostly restricted to freshwater environments, but all three major genera of medical importance (*Aedes, Anopheles*, and *Culex*) include both freshwater and saltwater species (Coluzzi and Sabatini 1969, Bradley 1987, Jude et al. 2012). In the genus *Aedes,* which includes the vectors of important arboviruses such as dengue, chikungunya, and Zika, all salt-tolerant species are not disease vectors (e.g., *Aedes detritus*, *Ae. campestris*, or *Ae. taeniorhynchus*), whereas all disease vectors (e.g., *Ae. aegypti* and *Ae. albopictus*) are considered obligate freshwater species (Ramasamy et al. 2011). However, it was recently reported that *Ae. aegypti* pre-imaginal stages can develop in brackish waters in Sri Lanka (Jude et al. 2012) and Brazil (de Brito-Arduino et al. 2015). Salinity tolerance changes in larvae of these invasive vector species may allow expanding their ecological niche and geographical distribution and could be another potential mechanism to promote their long-range dispersal. Consequently, salinity tolerance changes in these species could influence the epidemiology of several arboviruses. This is particularly important because areas contaminated by brackish waters are likely to expand in the future due to the global warming-induced rise in sea levels (Ramasamy and Surendran 2011).

Another example of the important consequences of salinity tolerance changes on disease transmission is found in the *Anopheles gambiae* species complex, which includes the main vectors of malaria and filariasis in Africa. Among the eight isomorphic species that constitute the complex, some are salt-tolerant (*An. melas*, *An. merus*, and *An. bwambae*), while others are obligate freshwater species (*An. gambiae*, *An. coluzzii*, *An. arabiensis*, *An. quadriannulatus*, and *An. amharicus*) during the larval phase (White et al. 2011). The salt-tolerant species are generally poor vectors, whereas the freshwater species include three of the most potent malaria vectors in sub-Saharan Africa. Consistent with their degree of salinity tolerance, salt-tolerant species have a more limited distribution, confined to mangrove coastal areas, salt-pans, or mineral geothermal springs, although all species of the complex can complete development and even prefer to oviposit in freshwater (Giglioli 1964, Mosha and Mutero 1982). It was recently reported that differences in salinity tolerance may also underlie habitat segregation between the closely related freshwater siblings *An. gambiae* (mainly inland) and *An. coluzzii* (some populations are confined to coastal areas; Tene Fossog et al. 2015). Therefore, larval tolerance of salinity constitutes a major physiological trait that characterizes the ecological niche of these species, and may be pivotal to adaptive radiation and speciation that have occurred or are still undergoing in this complex.

Breeding sites of these mosquitoes are alternately prone to dilution by rain, and to salinity increase by evaporation or flooding of coastal marshes. Moreover, human activities can influence the amount of salts in breeding sites by modifying coastal habitats (Ramasamy and Surendran 2011), polluting urban breeding sites (Tene Fossog et al. 2013), or by using deicing salts (Muñoz et al. 2015, Kaushal et al. 2018, Schuler and Relyea 2018). This last action has been largely neglected, but has important consequences in temperate countries where desalination is regularly used for anti-icing or deicing pavements and roads, and could contribute to increase the salt concentration in freshwater bodies. Presumably, the larvae of these mosquitoes respond to these changing conditions through osmoregulation. Therefore, *Aedes* and *Anopheles* species are not only important vectors of pathogens, but also good models for comparative studies on the predictive physiological bases of tolerance to salinity. In 1972, Bayly discussed an attempt at establishing a relationship between the osmoregulatory ability and salinity tolerance in salt-tolerant and hyper-iso-osmoregulating freshwater organisms (Bayly 1972). The author noted that if the upper limit of salinity tolerance occurs when the hemolymph osmolality reaches the isosmotic line or is slightly above this point, the osmotic pressure rankings in freshwater should be conserved, thereby providing a way to infer the salinity tolerance rankings from the osmotic pressure in freshwater. However, in mosquitoes, it is not known whether osmotic pressure rankings in diluted freshwater are conserved. In this work, we assessed the relationship between osmoregulatory ability and degree of salinity tolerance in larvae of six laboratory-reared mosquito species that belong to the three major mosquito genera (*Aedes*, *Anopheles*, and *Culex*) and are all major vectors of disease and obligate freshwater species. We hypothesized that species with higher osmoregulatory capacity should also be able to tolerate higher salinities. We quantified the larval hemolymph osmotic responses in varying salinity conditions to assess their osmoregulatory ability, and the acute dose–mortality response to increasing salinity concentrations to establish the degree of salinity tolerance, expressed by the median lethal concentrations. We then assessed the correlations between median lethal concentrations and hemolymph osmolality.

Methods

Mosquito larvae

In this study, multigenerational laboratory colonies of mosquito larvae were used (Table 1). Since their establishment at the IRD (Institut de Recherche pour le Développement, France) insectarium in Montpellier, all strains have been maintained in standard insectarium conditions in a controlled environment: 27°C, 80% relative humidity, and 12-h:12-h light/dark cycle. Larvae were reared in plastic trays (27 × 20 × 6 cm) filled with 1 liter (L) of deionized freshwater (DW) and fed 100 mg/d of finely ground fish meal (TetraMin). Fourth-instar larvae were

Table 1. Mosquito colonies used in the bioassays.

Species	Colony name	Salinity habitat preference	Geographic origin	Year collected
Aedes aegypti	Bora	Fresh–subsaline water	Bora Bora, French Polynesia	1990
Aedes albopictus	Nice	Fresh–subsaline water	EID, France	2012
Anopheles coluzzii	Yaounde	Fresh–polluted water	Yaoundé, Cameroon	1988
Anopheles gambiae	Kiss	Freshwater	Kisumu, Kenya	1975
Culex quinquefasciatus	S-Lab	Fresh–polluted water	Georghiou et al.	1966
Culex pipiens	Cupp	Freshwater	Cévenne, France	2013

selected for the experiments to optimize hemolymph sampling. Before the assays at all salinities (see *Salinity tolerance assays*), larvae were fed to avoid cannibalism, and to standardize as much as possible their physiological status. For assays in DW, two groups were used: fed group and group not fed for 24 h prior to tests.

Experimental media

Test media with decreasing salinity ($n = 5$) were prepared by adding DW to natural seawater (SW) collected offshore of Palavas-les-Flots, France (~34 ppt, 1000 mOsm/kg, considered as 100% seawater), that was the stock solution. Salinity was expressed as osmolality (in mOsm/kg) and as salt content of the medium (in ppt); 3.4 ppt is equivalent to 100 mOsm/kg. The SW percentage and salt concentration of the five test media were 50% (17 ppt), 40% (13.6 ppt), 30% (10.2 ppt), 20% (6.8 ppt), and 10% (3.4 ppt). Five liters of each dilution was prepared, stored in a dark room at 4°C, and used at 27°C for the survival and osmoregulation experiments. Also, two control treatments were set up: 100% DW without food (0 mOsm/kg) and 100% DW with food added (~5 mOsm/kg). The osmotic pressure of test media was measured in a 20-µL aliquot of each medium with a micro-osmometer (Model 3320; Advanced Instruments, Norwood, Massachusetts, USA).

Salinity tolerance assays

All assays were carried out in an incubator at 27°C. Approximately two hundred 4th-instar larvae of each species were placed in plastic trays containing 1 L of DW and fed for 2 h as described above. Twenty larvae were then placed in covered plastic cups containing 100 mL of test medium (DW without food, DW with food added, 10%, 20%, 30%, 40%, and 50% of SW). Each assay with twenty larvae was replicated two to four times for each species, depending on larval availability. After 24-h exposure, larval mortality was quantified. All larvae that did not display an escape response (swimming and/or diving) or did not move upon stimulation with a pipette tip were scored as dead. Surviving larvae were prepared for hemolymph osmolality measurements (see *Osmoregulation*).

Assessment of acclimation time

Pilot trials were carried out with *An. gambiae* and *Ae. albopictus* larvae to assess the acclimation time needed to adjust their hemolymph osmolality to a stable value when switched from DW to diluted SW. This experiment was necessary to determine the experimental exposure period for assessing the larval osmoregulatory ability in response to varying salinities (see *Osmoregulation*). Larvae maintained since hatching in DW were rapidly transferred to 30% SW, and the hemolymph osmolality of the surviving larvae was measured (see technique below) at T0 (DW) and then at 1, 2, 3, 6, 12, and 24 h of exposure to SW.

Osmoregulation

The larval osmoregulatory capacity in the presence of different SW percentages was assessed using the survivors of the salinity tolerance assays. To measure the hemolymph osmolality, larvae were gently captured with a pipette immediately after the test, superficially dried on filter paper, and quickly immersed in mineral oil to prevent evaporation and desiccation. The remaining adherent water was removed using a glass micropipette under a dissecting microscope. A new micropipette was then inserted dorsally between thorax and abdomen into the hemocoel (League et al. 2015) to collect hemolymph samples (about 30 nanoliter, nL) that

were then measured with reference to an internal standard of 300 mOsm/kg on a Kalber-Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, New York, USA).

Data analysis

The dose–mortality response to salinity of each species was analyzed by logistic regression. After verification that the dose response was significantly different among species, the median lethal concentration (LC_{50}) of each species was estimated with the function dose.p of the MASS library in R (R Core Team 2017). To test the statistical significance of differences between species in the SW concentration–mortality response and hemolymph osmolality, GLMM with logit link functions and binomial errors were fitted to the whole data using the LME4 library and the glmer function. The number of dead specimens at SW concentration was used as fixed effect and each biological replicate as random effect. The goodness-of-fit was compared using the Akaike information criterion (AIC). Statistical correlations between hemolymph osmolality and tolerance to salinity were computed using the cor function and the Pearson method in the STATS library in R (R Core Team 2017). Figures were plotted with the ggplot2 library (Wickham 2009).

Results

Salinity tolerance

Survival of the six different mosquito species after 24-h exposure to the test media is summarized in Fig. 1. No mortality was observed in DW, with and without food, in any of the six species. Mortality increased starting from 200 or 300 mOsm/kg (6.8–10.2 ppt) in function of the species. With the exception of *Ae. albopictus*, 100% mortality was observed between 300 and 500 mOsm/kg (10.2–17 ppt). The LC_{50} values ranged from 248 ± 7 to 510 ± 16 mOsm/kg (8.4–17.3 ppt; Table 2). *Ae. albopictus,* the tiger mosquito, was the most salt tolerant species, followed by *An. coluzzii,* and then *Ae. aegypti*, *Cx. quinquefasciatus*, *An. gambiae*, and *Cx. pipiens* (the least tolerant species). However, only *Cx. quinquefasciatus* mortality was significantly

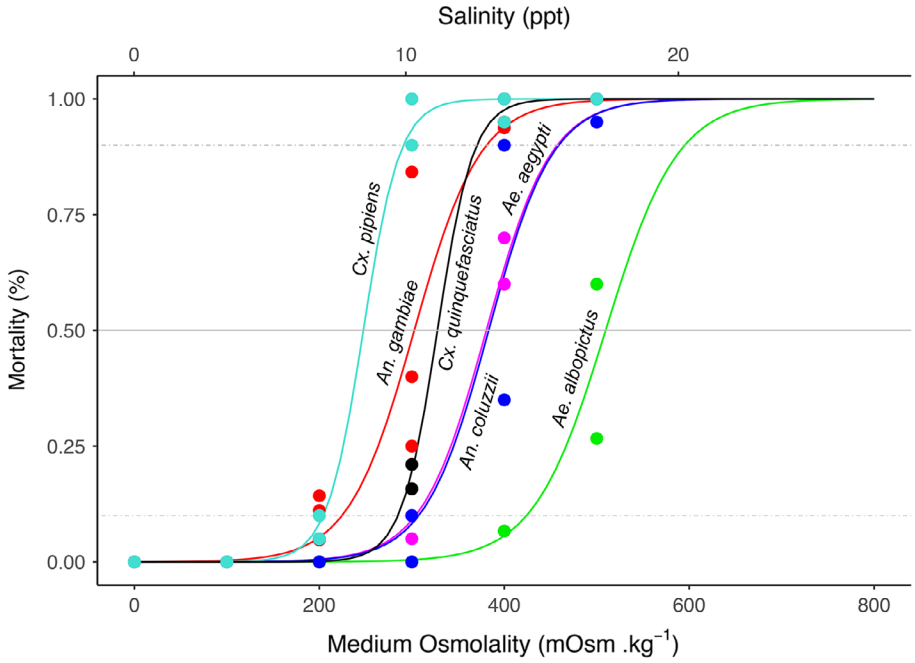

Fig. 1. Survival of larvae of six mosquito species exposed to an acute salinity stress. Each point represents one replicate. Dose–response mortality curves were fitted by logistic regression, to assess the degree of salinity tolerance of each species.

Table 2. LC_{50} and hemolymph osmolality values at different salinities in larvae of six mosquito species.

Species	LC^{50} (mOsm/kg) (ppt)	Hemolymph osmolality (mOsm/kg) in media of osmolality/salinity						
		DW (0) 0 ppt	DW + food (~5) 0.17 ppt	100 3.4 ppt	200 6.8 ppt	300 10.2 ppt	400 13.6 ppt	500 17.0 ppt
Aedes aegypti	381 ± 8	239 ± 15	269 ± 12	278 ± 12	305 ± 11	366 ± 13	414 ± 5	
	12.95 ppt	(10)	(10)	(10)	(10)	(10)	(5)	
Aedes albopictus	510 ± 16	238 ± 21	271 ± 14	270 ± 7	292 ± 9	326 ± 8	410 ± 12	530 ± 15
	17.3 ppt	(10)	(10)	(10)	(10)	(10)	(10)	(10)
Anopheles coluzzii	383 ± 9	226 ± 13	242 ± 15	274 ± 11	292 ± 19	343 ± 15	420 ± 28	
	13.0 ppt	(10)	(10)	(10)	(9)	(8)	(8)	
Anopheles gambiae	304 ± 7	216 ± 13	237 ± 11	274 ± 25	300 ± 8	346 ± 8	415 ± 7	
	10.3 ppt	(10)	(10)	(10)	(10)	(10)	(2)	
Culex pipiens	248 ± 7	203 ± 9	226 ± 8	222 ± 12	218 ± 8	320 ± 10		
	8.4 ppt	(10)	(10)	(10)	(10)	(3)		
Culex quinquefasciatus	328 ± 6	223 ± 13	260 ± 9	261 ± 9	250 ± 9	323 ± 7		
	11.2 ppt	(10)	(10)	(10)	(10)	(10)		

Notes: Each point represents the mean value ± SD, with the number of sampled animals in brackets. Approximate values in ppt of LC_{50} and of the test medium salinity are given.

different compared with the other species (GLMM, $P < 0.05$). When considering the salinity level as a factor, the mortality of *Cx. pipiens* and C*x. quinquefasciatus* was significantly different compared with the other species (GLMM, $P < 0.05$).

Acclimation time

After the rapid transfer of *An. gambiae* and *Ae. albopictus* larvae from DW to 300 mOsm/kg test medium, hemolymph osmolality increased gradually before stabilization between 3 and 6 h for both species (Fig. 2). In all subsequent experiments, larvae were kept in each test medium for 24 h before sampling.

Osmoregulation

The results of the osmoregulation experiments are presented as hemolymph osmolality values in function of the test medium osmolality (Fig. 3, Table 2). Overall, the osmoregulation profile was similar in all species of the three genera *Aedes*, *Anopheles*, and *Culex*: All were hyper-iso-osmoregulators (i.e., hyper-regulators in DW and at low salinities, and osmoconformers at higher salinity). However, significant differences were observed among species.

In DW without food, all species were hyper-regulators, and hemolymph osmolality was significantly different among species ($F = 8.70$, df = 5.54; $P < 0.0001$). Hemolymph osmolality varied (mean ± SD) between 203 ± 9 and 239 ± 15 mOsm/kg. *Ae. aegypti* displayed the highest osmolality (239 ± 15 mOsm/kg; very close to *Ae. albopictus*, 238 ± 21 mOsm/kg) followed by *Ae. albopictus*, *An. coluzzii*, *Cx. quinquefasciatus*, *An. gambiae*, and *Cx. pipiens* (203 ± 9 mOsm/kg; Table 2). Post hoc contrast analysis (Tukey's honestly significant difference at $P > 0.05$) identified three groups that included mosquito species with non-significant differences in osmolality: (1) *Ae. aegypti*, *Ae. albopictus*, *Cx. quinquefasciatus*, and *An. coluzzii*; (2) *Cx. quinquefasciatus*, *An. coluzzii*, and *An. gambiae*; and (3) *An. gambiae* and *Cx. pipiens*.

In DW supplemented with food, hemolymph osmolality was higher (on average by 27 ± 5 mOsm/kg) than in DW without food, independently of the species considered ($F = 1.44$, df = 5.113; $P = 0.215$).

In test media with osmolality values of ~5 (DW with food; 0.17 ppt) and 200 mOsm/kg (6.8 ppt), the hemolymph osmolality of the six species remained stable or increased slightly by 20–60 mOsm/kg. In test media with osmolality higher than 200 mOsm/kg (6.8 ppt), hemolymph osmolality increased in all species until hyper-osmoconformation (i.e., hemolymph osmolality only 10–20 mOsm/kg higher than the medium osmolality). This condition was reached at 200 mOsm/kg (6.8 ppt) in *Cx. pipiens*, 300 mOsm/kg (10.2 ppt) in *Cx. quinquefasciatus*, and 400 mOsm/

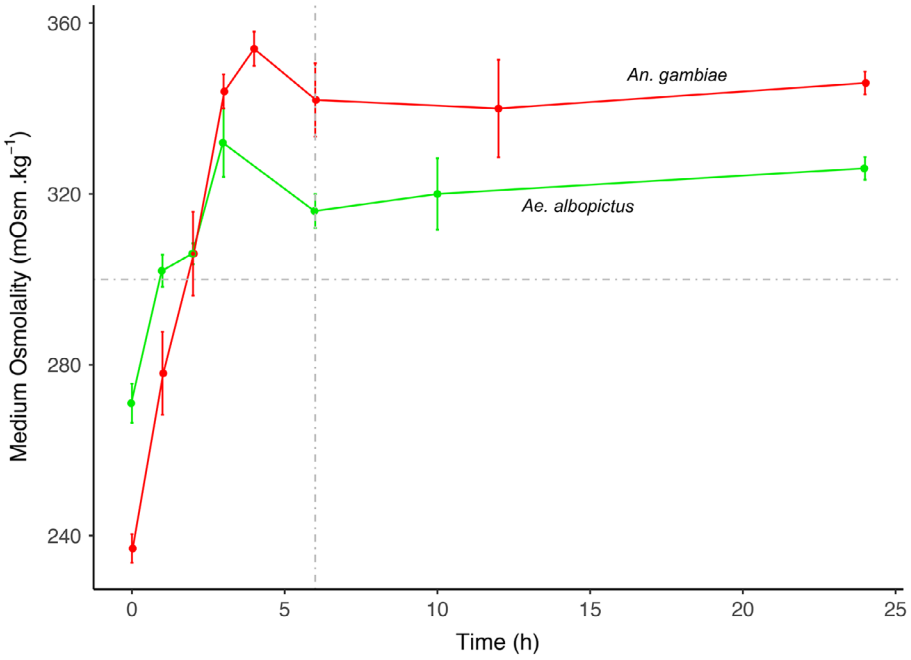

Fig. 2. Variations in hemolymph osmolality of *Anopheles gambiae* (red) and *Aedes albopictus* (green) larvae following rapid transfer from deionized water (DW) to a 300 mOsm/kg medium. The curves represent the mean value ± SD from 5 animals, and 10 animals for the lowest and highest osmolality values.

kg (13.6 ppt) in *Ae. aegypti*, *Ae. albopictus*, *An. coluzzii*, and *An. gambiae*. *Ae. albopictus* was the only species with specimens still alive and showing hyper-osmoconformation at 500 mOsm/kg (17 ppt).

Correlations between larval salinity tolerance and osmoregulation

Analysis of the correlation between hemolymph osmolality and salinity tolerance (expressed by the median lethal concentration) in larvae of the six species (scattergram in Fig. 4) showed a strong and significant correlation between hemolymph osmolality in DW and salinity tolerance (Pearson $r = 0.87$; $P < 0.05$). This correlation progressively decreased when considering hemolymph osmolality at higher salinities (Appendix S1).

Discussion

Salinity tolerance

This study investigated the salinity tolerance in larvae of six laboratory strains of the major vector mosquitoes belonging to three different genera: *Ae. albopictus* (the most tolerant to salinity), *An. coluzzii*, *Ae. aegypti*, *Cx. quinquefasciatus*, *An. gambiae*, and *Cx. pipiens* (the least tolerant to salinity). *Ae. albopictus* was the only species with survivors after 24-h incubation in test medium with 500 mOsm/kg (50% SW). *Ae. albopictus* and *An. coluzzii* phenotypic plasticity to colonize a variety of habitats was already known (Ramasamy et al. 2011). In our study, death of larvae began from 20% SW (200 mOsm/kg, 6.8 ppt), as previously reported (White et al. 2013). Except for *Cx. pipiens*, all studied species could survive in brackish water below 30% of SW (10.2 ppt; *An. gambiae*, *Cx. quinquefasciatus*), 40% of SW (13.6 ppt; *An. coluzzii*, *Ae. aegypti)*, and 50% of SW (17 ppt; *Ae. albopictus*). Previous studies on the salinity tolerance of *Ae. aegypti* and *Ae. albopictus* showed that the LC_{50} ranged from 11.9 to 16 ppt, depending on the medium origin (fresh or brackish water-derived) and instar of larvae (Ramasamy and Surendran 2011). Recently, White et al. (2013) found that the larvae of the freshwater members of the *Anopheles gambiae* complex cannot survive after 24 h at this level of salinity following direct transfer from

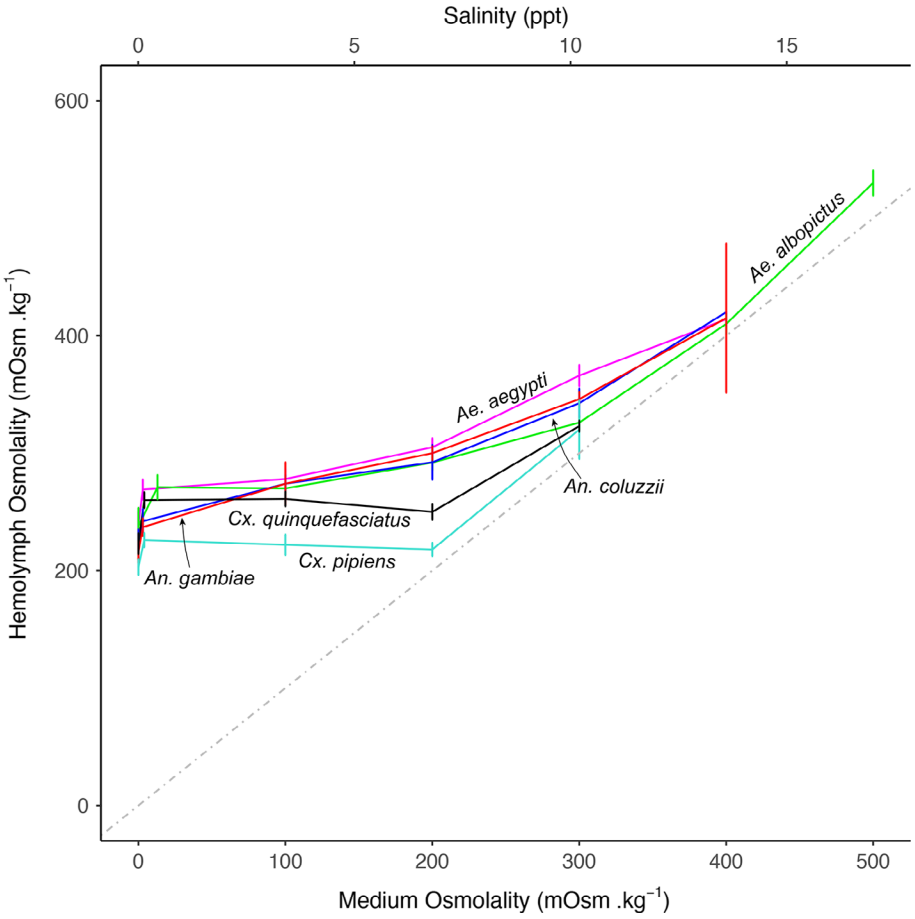

Fig. 3. Variations in hemolymph osmolality of larvae of six mosquito species in function of the external medium osmolality. The isosmotic line is drawn. Each point represents the mean value ± SD from 10 animals, and 2 to 5 animals at the highest osmolality values (400 and 500 mOsm/kg).

freshwater. These authors also found that the LC_{50} was 33–36% of SW for *An. coluzzii* and 33–38% for *An. gambiae*, compared with 38% and 30%, respectively, in our study. This slight difference could be explained by methodological differences (NaCl solutions in the study by White et al. vs. diluted SW in our study) or from differences between strains of the same species. However, tolerance to salinity may vary in time due to the cost of osmoregulation mechanisms and salinity-induced oxidative stress (Rivera-Ingraham and Lignot 2017). Indeed, slight differences between studies might be expected due to the strain origins. In our study, multigenerational colonies of the six major vectors were used to investigate the potential relationship between salinity tolerance and osmoregulation. However, extrapolation of our results to natural populations must be done with caution because many traits can be unpredictably modified during laboratory maintenance for many generations. For instance, in *Ae. aegypti* from Australia, most of the measured fitness traits were comparable between natural and laboratory-reared strains, thus not consistent with a local adaptation to insectary conditions (Ross et al. 2019). On the other hand, inbreeding depression in established colonies of some *Anopheles* species affects fecundity or genetic diversity (Ekechukwu et al. 2015, Lainhart et al. 2015). Nevertheless, despite the potential bias of using laboratory colonies, our study provides evidence of differences in salinity tolerance across freshwater mosquito species.

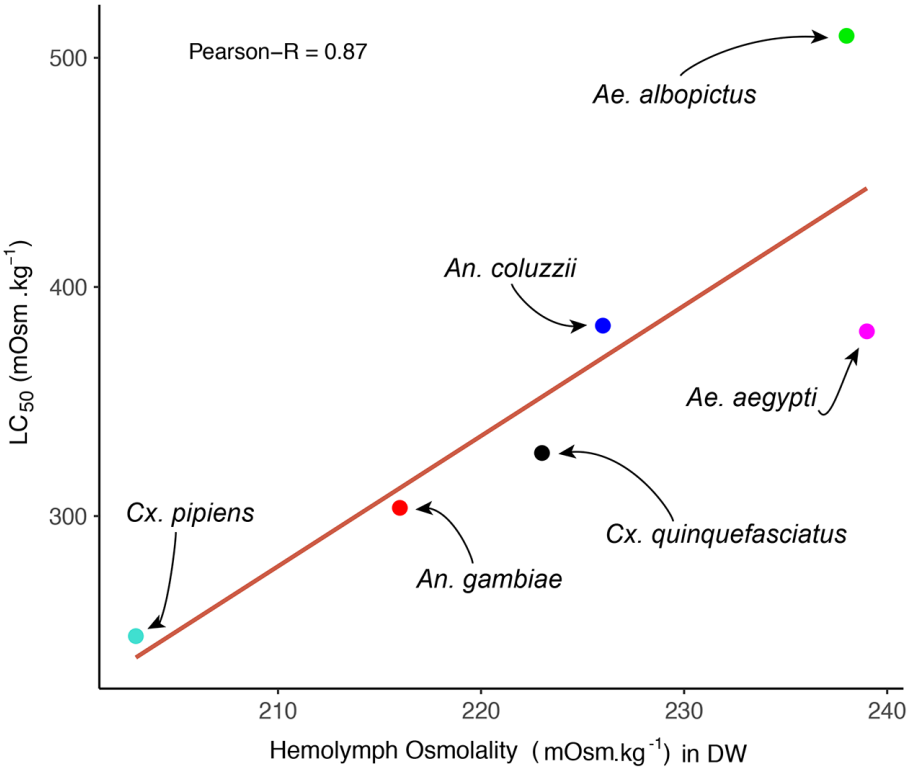

Fig. 4. Correlation between larval salinity tolerance and hemolymph osmolality in six mosquito species. Scattergram of the relationship between hemolymph osmolality in deionized water (DW) and the median lethal salinity concentration.

Acclimation time

In our study, after rapid transfer from DW (0 mOsm/kg) to 300 mOsm/kg (10.2 ppt) water, the time required for osmotic stabilization in *An. gambiae* and *Ae. albopictus* fourth-instar larvae ranged from 3 to 6 h. These times are higher than those generally found (1–2 h) in larval crustaceans (Charmantier 1998). In first-instar juveniles of the freshwater crayfish *Astacus leptodactylus*, acclimation time was 6 h (Susanto and Charmantier 2000), but the salinity gradient was double (from freshwater to 611 mOsm/kg, 20.8 ppt) than in our experimental setting, and animals were bigger (9–11 mm long, 3–4 mm carapace width) than our mosquito larvae (4–7 mm long, 0.4–0.6 mm abdominal width), the size of which is similar to the size of most larval crustaceans. More accurate measurements of comparative surface-to-volume ratios should be made between groups. The longer acclimation time in larval mosquitoes could be explained by (1) the lower permeability of the insect cuticle to water and ions that limits passive exchanges, compared with the crustacean cuticle; (2) different drinking rates between mosquito larvae and other groups (Bradley and Phillips 1977, Patrick and Bradley 2000); and/or (3) more rapid involvement of cellular and molecular osmoregulatory mechanisms in crustaceans. This third hypothesis is less likely, given the slow development of osmoregulatory organs in larval crustaceans (Charmantier 1998).

Osmoregulation

Our analysis of the osmoregulatory responses of laboratory strains of six major mosquito vectors that breed in different natural ecological niches shows that all studied species can hyperosmoregulate in diluted SW medium with osmolality up to 200–300 mOsm/kg (6.8–10.2 ppt), although with species-related differences. Hyperregulation is a universal adaptation strategy by

freshwater organisms (Bradley 2009) to maintain the osmolality of extracellular fluids between 200 and 300 mOsm/kg in most species (Evans and Claiborne 2009). In DW, hemolymph osmolality ranged between 215 and 240 mOsm/kg in larvae of the five of the studied mosquito species, with a lower value (~200 mOsm/kg) in *Cx. pipiens*. This hyper-osmoregulatory response that maintains hemolymph concentration within a range close to the typical osmolality of insect hemolymph was previously reported in freshwater mosquito larvae (Chown and Nicolson 2004, Bradley 2009). Particularly, in *Ae. aegypti* and *Cx. pipiens* kept in freshwater, Wigglesworth (1938) found osmolality values of ~222 mOsm/kg in unfed larvae and of ~270 mOsm/kg in fed larvae (evaluated on a graph and calculated from the percentage of NaCl used as units, with 1% of NaCl equivalent to 318 mOsm/kg). This author did not report differences between species, differently from our study, but the *Ae. aegypti* osmolality values are very similar between studies (in freshwater unfed/fed: 222/270 mOsm/kg in Wigglesworth (1938); 239/269 mOsm/kg in this study). Similar values of hemolymph osmolality were reported for most freshwater invertebrates at different phases of post-embryonic development, with values below 200 mOsm/kg generally considered as indicating week physiological conditions (Charmantier et al. 2009). In contrast, higher osmolality (up to 400 mOsm/kg) was observed in a few strong freshwater osmoregulators, such as crayfish (Charmantier et al. 2009), and much lower hemolymph osmolality (down to 50 mOsm/kg) in few freshwater mollusks (Deaton 2009). In freshwater teleosts, blood osmolality is 260–300 mOsm/kg (Evans and Claiborne 2009). Thus, a value of 200–300 mOsm/kg appears as an optimum osmolality level for extracellular fluids in most freshwater animals. In our study, hemolymph osmolality was about 20 mOsm/kg higher in larvae exposed to DW supplemented with food than in DW without food. Wigglesworth (1938) also reported up to 50 mOsm/kg higher osmolality values in well-fed larvae. This difference could be explained by the presence of ions and organic osmolytes in the medium with food that may be taken up by the organism. In test media with osmolality and salinity values of 200–300 mOsm/kg (6.8–10.2 ppt), mosquito larvae were hyper-osmoconformers. This indicates that they do not possess hypo-osmoregulation mechanisms, differently from other insects living in saline waters, such as Hydrophilidae (Dytiscidae, Coleoptera) that can hypo-osmoregulate (Pallarés et al. 2015). The slightly higher hemolymph osmolality compared with the test medium in these conditions is probably due to organic osmoeffectors in the hemolymph, as shown in species of the *Culex* genus that osmoconform by accumulating organic compounds, such as proline and trehalose, in their hemolymph (Bradley 1987, 1994, Patrick and Bradley 2000).

In insects, the hyper-osmoregulation mechanism involves production of diluted urine by the Malpighian tubule system and rectum (to compensate for water entering the body) coupled with the replacement of lost salts by active ion uptake (Bradley 1987, Beyenbach and Piermarini 2009, White et al. 2013). Such adaptation could be involved in the osmoregulatory responses observed in our study. Water and osmotic homeostasis are under hormonal control, and the required biological processes are very different at morphological, biochemical, and cellular levels among insect groups with different ecological requirements (Larsen et al. 2014). Despite the potential bias of using laboratory colonies, our analysis starts to address the physiological basis of this ecological trait that should be now investigated in natural populations of mosquito vectors. While this study does not allow addressing the mechanistic aspects of ionic exchanges, the osmotic regulation patterns reported here provide a basis for comparative studies on the osmoregulatory mechanisms in a wide range of freshwater vector mosquitoes.

Relationship between larval salinity tolerance and osmoregulation

It is known that hemolymph osmolality increases with increasing concentration of the incubation medium, with rates differing according to the species osmoregulation ability, from hyper-osmoregulators to osmoconformers (Evans and Claiborne 2009). Despite the laboratory origin of our species, we observed the same pattern with species-specific variations. These results are in agreement with the hypothesis that different osmoregulatory capacities in species mediate their habitat segregation across a salinity gradient, as

observed in crustaceans (Charmantier et al. 2009) and insects (Céspedes et al. 2013, Pallarés et al. 2015), including mosquitoes (Edwards 1982). Moreover, our study evaluated the correlation between LC_{50} of each species and hemolymph osmolality in the different test media. In DW, species with high LC_{50} had a high hemolymph osmolality, and those with low LC_{50} had low hemolymph osmolality with a strong correlation between these sets of data (Fig. 4). This correlation was particularly clear at the two extremes: low osmolality, low LC_{50} in *Cx. Pipiens*, and high osmolality, high LC_{50} in *Ae. albopictus*. A relationship between salinity tolerance and osmoregulatory capacity was observed also in other insect groups. For instance, an elegant study on eight species from two genera of Hydrophilidae (Dytiscidae, Coleoptera) that have invaded saline waters independently from freshwater ancestors showed that at higher salinities, freshwater species are conformers and saline water species are hyporegulators (Pallarés et al. 2015). Although this study did not show a correlation between osmoregulatory capacity and survival in freshwater, it suggested that the different osmotic capacities among species might mediate their differential tolerances to salinity, and consequently, their habitat segregation across the salinity gradient. Conversely, this relationship between salinity tolerance and osmoregulatory capacity is not present in the mayfly nymph *Austrophlebioides pusillus* (Ephemeroptera) that dies upon rises in salinity that are well below the iso-osmotic point, an exception among freshwater invertebrates (Dowse et al. 2017). Our results based in long-term mosquito colonies show a significant correlation between salinity tolerance and osmoregulation; however, other species and natural populations should be tested in additional studies.

Conclusion

Despite the laboratory origin of the six studied mosquitoes species, the strong physiological correlation between hemolymph osmolality and salinity tolerance suggests a conserved physiological response across species. However, local adaptation may play an important role in the variations of the osmoregulation capacity of different populations. During the last decades, human activities, such as urban pollution (Tene Fossog et al. 2013), coastal habitat modification (Ramasamy and Surendran 2011), and use of deicing salt on pavements and roads, have affected the salt concentration in natural habitats (Dugan et al. 2017, Hintz and Relyea 2017, Kaushal et al. 2018). Our laboratory results may help to unravel how variations in salinity tolerance among different natural populations of disease-vector mosquito species contribute to local adaptation and to their eco-geographical distribution. The physiological mechanisms of the relationship between hemolymph osmolality and salinity tolerance constitute an area for future research in natural conditions.

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Supporting Information

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.2783/full