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RESEARCH ARTICLE

Dose and developmental responses of *Anopheles merus* larvae to salinity

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SUMMARY

Saltwater tolerance is a trait that carries both ecological and epidemiological significance for *Anopheles* mosquitoes that transmit human malaria, as it plays a key role in determining their habitat use and ecological distribution, and thus their local contribution to malaria transmission. Here, we lay the groundwork for genetic dissection of this trait by quantifying saltwater tolerance in three closely related cryptic species and malaria vectors from the Afrotropical *Anopheles gambiae* complex that are known to differ starkly in their tolerance to salinity: the obligate freshwater species *A. gambiae* and *A. coluzzii*, and the saltwater-tolerant species *A. merus*. We performed detailed comparisons of survivorship under varying salinities, using multiple strains of *A. gambiae*, *A. coluzzii* and *A. merus*, as well as F1 progeny from reciprocal crosses of *A. merus* and *A. coluzzii*. Additionally, using immunohistochemistry, we compared the location of three ion regulatory proteins (Na^+/K^+-ATPase, carbonic anhydrase and Na^+/H^+-antiporter) in the recta of *A. coluzzii* and *A. merus* reared in freshwater or saline water. As expected, we found that *A. merus* survives exposure to high salinities better than *A. gambiae* and *A. coluzzii*. Further, we found that exposure to a salinity level of 15.85 g $NaCl\,l^{-1}$ is a discriminating dose that kills all *A. gambiae*, *A. coluzzii* and *A. coluzzii–A. merus* F1 larvae, but does not negatively impact the survival of *A. merus*. Importantly, phenotypic expression of saltwater tolerance by *A. merus* is highly dependent upon the developmental time of exposure, and based on immunohistochemistry, salt tolerance appears to involve a major shift in Na^+/K^+-ATPase localization in the rectum, as observed previously for the distantly related saline-tolerant species *A. albimanus*.

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Key words: *Anopheles coluzzii*, *Anopheles gambiae*, *Anopheles merus*, DAR cells, ion transport, malaria vector, mosquito, Na^+/K^+-ATPase, osmoregulation, salinity tolerance.

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INTRODUCTION

Mosquitoes (Diptera: Culicidae) are a monophyletic family comprising more than 3500 species that occupy every continent except Antarctica (Harbach, 2011). The immature stages occur in a diverse array of aquatic environments (Harbach, 2007), but the vast majority (95%) are restricted to freshwater (water with an osmotic concentration less than that of larval hemolymph, ~300 osmol l^{-1} or ~30% seawater) (Bradley, 1987). Only 5% of mosquito species have larvae capable of surviving in the salty waters of coastal marshes, mangrove swamps, tide pools, hypersaline ponds and mineral springs (O'Meara, 1976; Clements, 1992). Given that the most closely related insect families contain only obligate freshwater species, it is likely that restriction to freshwater is the ancestral condition in Culicidae, and saltwater tolerance is a derived trait (Bradley, 2008; Albers and Bradley, 2011). Although relatively rare in mosquitoes, saltwater tolerance occurs in 10 different genera and thus appears to have evolved repeatedly in independent mosquito lineages (O'Meara, 1976; Bradley, 2008; Albers and Bradley, 2011). Saltwater-tolerant mosquito larvae can develop normally in both fresh and saline habitats, but saltwater preference may be adaptive because of high nutrient levels coupled with reduced competition and predation in brackish or saline sites, particularly those sites subject to rapid changes in salinity (Bradley, 2008).

To survive in water that differs in osmolarity from their hemolymph, mosquito larvae must regulate their absorption and excretion of ions. In saline water, the challenge is to counteract salt gain. The repeated evolution of salt tolerance in different mosquito genera has involved distinct morphological and physiological solutions to that challenge. By accumulating organic compounds such as proline and trehalose in the hemolymph, species in the *Culex* and *Culiseta* genera osmoconform, increasing their osmolarity to match external concentrations (Bradley, 1987; Bradley, 1994; Patrick and Bradley, 2000). Many other salt-tolerant culicine and anopheline species osmoregulate rather than osmoconform, a function accomplished primarily by the rectum, although Malpighian tubules and anal papillae also play a role (Koch, 1938; Copeland, 1964; Bradley, 1987; Coetzee and Le Sueur, 1988; Clements, 1992; Xiang et al., 2012). In freshwater, the rectum counteracts salt loss by ion resorption from the primary urine; in saline water, the rectum counteracts salt gain by excreting a hyperosmotic urine (Bradley, 1987; Clements, 1992; Bradley, 1994). However, rectal morphology differs significantly between the culicine and anopheline mosquito subfamilies (Bradley, 1987; Bradley, 1994). In culicines, the larval recta of obligate freshwater species are structurally uniform; those of salt-tolerant species are divided into histologically distinct anterior and posterior segments, of which the anterior segment is

functionally similar to the recta of obligate freshwater culicines. Only the posterior rectal segment produces hyperosmotic urine. Thus, in culicines, salt tolerance is conferred by a dedicated structure and cell type not found in obligate freshwater species. By contrast, all anopheline species examined to date have structurally identical larval recta regardless of salt tolerance (Smith et al., 2008). The recta of anopheline larvae consist of two cell types, one (DAR cells: dorsal anterior rectum) forming the dorsal anterior ~25% of the organ, and the other (non-DAR cells) forming the remaining ventral anterior and posterior parts. A model of ion regulation by anopheline larvae proposes that the same non-DAR cells are used to excrete or resorb ions, depending upon alternative localization patterns of membrane energizing proteins (Smith et al., 2010). As such, the degree of salt tolerance in a given anopheline species depends upon the degree to which that species is capable of shifting the location of these proteins.

Considerable advances in understanding the physiological basis of saltwater tolerance in mosquitoes have not been matched by advances at the genetic level, owing to the dearth of genetic resources for mosquitoes until the last decade. The complete genome sequencing and ensuing reference genome assembly of the African malaria mosquito *Anopheles gambiae* Giles (Holt et al., 2002) rapidly transformed this species into a model system in which the genetic basis of ecologically and epidemiologically important phenotypes can be dissected. Saltwater tolerance is a trait that carries both ecological and epidemiological significance for *Anopheles* species that transmit human malaria, as it plays a key role in determining their habitat use and ecological distribution, and thus their local contribution to malaria transmission. Moreover, unlike other complex ecological, behavioral and life-history traits of epidemiological importance that are probably polygenic, saltwater tolerance is relatively tractable, likely governed by a few major loci with large effects, and straightforward to assay. The *Anopheles gambiae* complex – a group comprising *A. gambiae sensu stricto* and at least seven isomorphic and very closely related sibling species (Coluzzi et al., 1979; Coluzzi et al., 2002; White et al., 2011; Coetzee et al., 2013) – provides an ideal study system for understanding the physiological and genetic basis for saltwater tolerance, as it contains both saltwater-tolerant species and obligate freshwater species [including *A. gambiae s.s.*, formerly *A. gambiae* S molecular form and hereafter *A. gambiae*, as well as *A. coluzzii* Coetzee and Wilkerson, formerly *A. gambiae* M molecular form (Coetzee et al., 2013)]. Three species within the complex can complete development in water sources with elevated ion levels: *Anopheles melas* Theobald and *Anopheles merus* Donitz exploit brackish pools on the west and east coasts of the continent, respectively, while *Anopheles bwambae* White larvae are found in the mineral springs of a Ugandan national park where multiple ions are at high concentrations (Gillies and De Meillon, 1968; White, 1973; Mosha and Mutero, 1982; Harbach et al., 1997; Sinka et al., 2010). Here, we lay the groundwork for future genetic mapping of the saltwater tolerance trait through detailed comparisons of survivorship under varying salinities, using multiple strains of *A. gambiae*, *A. coluzzii* and *A. merus* (the only salt-tolerant species in the complex available in laboratory culture), and F1 progeny from reciprocal crosses of *A. merus* and *A. coluzzii*. Additionally, we compared the location of three ion regulatory proteins (Na^+/K^+-ATPase, carbonic anhydrase and Na^+/H^+-antiporter) in the recta of *A. coluzzii* and *A. merus* reared in freshwater or saline water. As expected, we found that *A. merus* survives exposure to high salinities better than *A. gambiae* and *A. coluzzii*. In addition, we found that exposure to a salinity level of 15.85 g $NaCl\,l^{-1}$ is a discriminating dose that kills all *A. gambiae*, *A. coluzzii* and *A. coluzzii–A. merus* F1 larvae, but does not negatively impact the survival of *A. merus*. Importantly, expression of salinity tolerance by *A. merus* is highly dependent upon the developmental time of exposure, and based on immunohistochemistry, salt tolerance appears to involve a major shift in Na^+/K+-ATPase localization in the rectum, as observed previously for the distantly related saline-tolerant species *A. albimanus* (Smith et al., 2008).

MATERIALS AND METHODS

Mosquito culture

The mosquito colonies used in this study are listed in Table 1. It is important to note that the *A. merus* MAF colony has been maintained since its inception in saline water (15.85 g $NaCl\,l^{-1}$), while the *A. merus* OPHANSI colony was adapted to deionized water soon after its establishment and has been cultured in deionized water ever since. All colonies were maintained in the University of Notre Dame insectary under controlled conditions of 27°C, 85% relative humidity and a 12 h:12 h light:dark cycle with 1 h dawn and dusk transitions. Larvae were reared in plastic trays (27×16×6.5 cm) with fitted lids, at a density of 200 l^{-1} of reverse-osmosis deionized water, unless otherwise stated. Each tray was fed 100 mg day^{-1} of a 2:1 mixture of finely ground fish pellets:baker's yeast for the first 3 days post-emergence, and 135 mg day^{-1} thereafter. For all experiments involving exposure to saline water, solutions of commercial sodium chloride (NaCl) were prepared with distilled water. For simplicity, we express the salinity of these solutions with reference to the approximate amount of dissolved NaCl in 100% seawater, assuming this is 31.7 g $NaCl\,l^{-1}$. Thus, our saline solutions ranging from 0% to 100% in increments of 10% contain: 0, 3.17, 6.34, 9.51, 12.68, 15.85, 19.02, 22.19, 25.3, 28.5 and 31.7 g $NaCl\,l^{-1}$.

Controlled interspecific crosses

Members of the *A. gambiae* complex can be reciprocally crossed in the laboratory to yield viable F1 hybrids (although male F1 hybrids are sterile) (Davidson et al., 1967). Toward eventual quantitative trait locus (QTL) mapping of salinity tolerance in *A. merus*, we performed preliminary reciprocal mass-crosses between

Table 1. Mosquito colonies used in this study

Species	Colony	MR4 no.	MR4 depositor	Geographic origin	Year collected
Anopheles coluzzii	Mali-NIH*	MRA-860	N. Besansky	Niono, Mali	2005
Anopheles coluzzii	Yaounde	N/A	N/A	Yaoundé, Cameroon	1988
Anopheles gambiae	Pimperena*	MRA-861	N. Besansky	Pimperena, Mali	2005
Anopheles gambiae	NDKO	N/A	N/A	Ndakoyo, Cameroon	2008
Anopheles merus	OPHANSI*	MRA-803	R. Maharaj	Natal, South Africa	1992
Anopheles merus	MAF*	MRA-1156	M. Coetzee	Kruger National Park, South Africa	1991

*Obtained through the MR4 as part of the BEI Resources Repository [National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIAID, NIH)].

the obligate freshwater *A. coluzzii* Mali-NIH and saltwater-tolerant *A. merus* MAF colonies to generate F1 hybrids and assess their salt tolerance relative to the parents. All aspects of F1 mosquito culture were as described above.

Acute salinity assays

To counter the tendency for *A. gambiae* egg cohorts to hatch asynchronously over several days or even weeks (Yaro et al., 2006; Kaiser et al., 2010), synchronous hatching was forced. To achieve this, eggs laid overnight by groups of females were introduced into rearing trays of deionized water lined with filter paper strips; trays were maintained in the insectary for 48 h. The filter paper strips with eggs adhering to them were then removed from the trays and placed onto paper towels to air dry at room temperature for ~5 min. Upon re-immersion in deionized water, the vast majority of eggs hatched instantly.

Immediately after hatching, replicate batches of 200 larvae from each of six colonies (Table 1) were counted, placed into plastic trays containing 1 l of deionized water, and reared under standardized conditions as described above until reaching the L4 stage. L4 larvae were transferred individually to wells of 96-well plates containing 250 µl of one of six saline solutions (0%, 10%, 20%, 30%, 40%, 50%). In addition, L4 larvae of *A. merus* MAF and OPHANSI colonies were transferred to a seventh saline solution (60%). Larvae were tested in replicates of four plates for each solution and each colony; in total, ~15,000 larvae were bioassayed. After 24 h of exposure, larval survival was scored by eye. Larval death was confirmed by repeated stimulation with a plastic pipette to test for a motile response.

Chronic salinity assays

For chronic bioassays, synchronous hatching was forced and replicate batches of 200 newly hatched larvae from each of six colonies were transferred to rearing trays containing 1 l of one of six solutions (ranging from 0% to 50% in increments of 10%). Larvae from both *A. merus* colonies were transferred to five additional saline solutions (from 60% to 100%). Larvae were maintained under standardized rearing conditions described above until death or pupation, which was scored as survival in this assay. Four replicates of 200 larvae were tested for each solution and colony; in total, ~37,000 larvae were bioassayed.

For the delayed exposure experiments, in which initial exposure to saline was delayed by 24, 48 or 72 h after hatching, larvae were reared in trays of deionized water for the duration of the delay before introduction to 50% salinity for their remaining development. Otherwise, methods matched the chronic salinity assays.

Immunolocalization

Cohorts of *A. merus* OPHANSI and MAF colonies were hatched and reared to the L4 stage under two alternative conditions: deionized water or 50% salinity. Similarly, a cohort of the *A. coluzzii* Mali-NIH colony was hatched and reared to the L4 stage in deionized water or 20% salinity, the maximum saline dose these larvae could tolerate without significant mortality. L4 larvae from both treatments of the three colonies were fixed by injection into the hemocoel of a 4% formaldehyde solution, then immersed in 4% formaldehyde and shipped to The Whitney Laboratory and processed further as described in detail previously (Smith et al., 2008). Briefly, larvae were transferred to Carnoy's solution for 90 min on ice and washed twice with 100% ethanol for 30 min each. Larvae were then cleared with aniline:methylaslicylate (1:1) overnight and embedded in paraffin. Sections of 6 µm were cut and mounted on gelatin-coated slides. Sections were deparaffinized, blocked with pre-incubation buffer and incubated simultaneously with three primary antibodies [to Na^+/H^+-antiporter and carbonic anhydrase (CA9) at a dilution of 1:1000, and Na^+/K^+-ATPase at a dilution of 1:10] in pre-incubation buffer. After three washes in TBS, sections were incubated simultaneously with three secondary antibodies (FITC-conjugated goat anti-rabbit, TRITC-conjugated goat anti-mouse and Cy5-conjugated donkey anti-chicken; Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:250 in pre-incubation buffer. After rinsing with TBS, slides were mounted in 60% glycerol in TBS with phenylenediamine (Sigma, St Louis, MO, USA). All images were captured using a Leica LSCM SP2 laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL, USA). Four to five representative recta were examined from each group.

Statistical analysis

The dose–mortality response to salinity by individual colonies was assessed through logistic regression in R (www.R-project.org). Median and 99% lethal concentrations (LC) were estimated for each

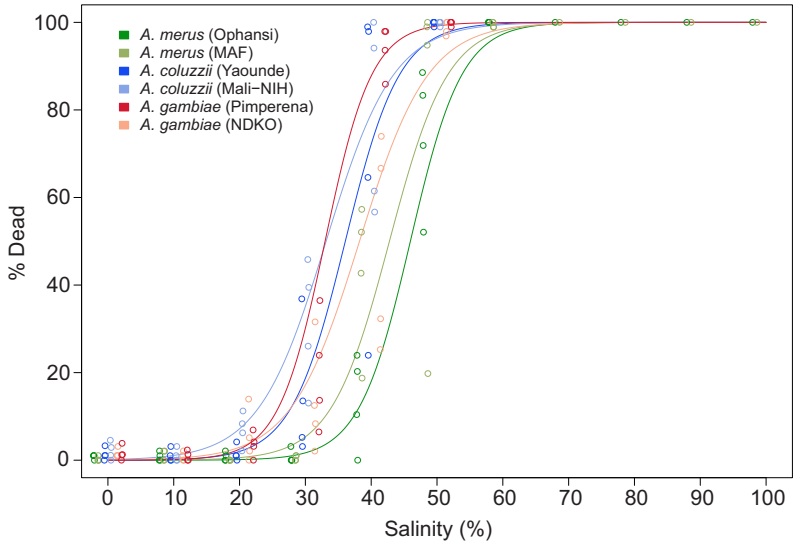

Fig. 1. Dose–mortality response to salinity measured for acute (24 h) exposure of 4th instar larvae (L4). Points denote observed mortality and are offset along the *x*-axis to aid readability. Logistic curves were individually fitted for each colony using all measured salinities.

Table 2. Median and 99% lethal salinity concentrations (LC; %) estimated for *A. coluzzii*, *A. gambiae* and *A. merus* after 24 h (acute) exposure of L4 larvae

Species (colony)	LC_{50}	LC_{99}	Slope
A. coluzzii (Yaounde)	36.1±2.30	55.0±6.50	0.242±0.077
A. coluzzii (Mali-NIH)	33.2±1.38	56.8±4.02	0.195±0.031
A. gambiae (NDKO)	38.2±1.57	62.2±4.84	0.192±0.036
A. gambiae (Pimperena)	33.0±2.86	49.4±7.91	0.281±0.125
A. merus (OPHANSI)	46.0±3.91	64.0±10.7	0.074±0.141
A. merus (MAF)	42.8±1.63	63.2±4.57	0.120±0.047

Data are given ±s.e.m.

colony using the *dose.p* function from the MASS library in R (Venables and Ripley, 2002). To test for the statistical significance of differences between species (and F1 hybrids) in the dose–mortality response, generalized linear models with logit link functions and quasibinomial errors were fitted to the whole data set, but because only *A. merus* colonies were exposed to salinities above 50% (a salinity level that invariably killed *A. gambiae* and *A. coluzzii*), *A. merus* data from salinities >50% were excluded. Similarly, models of the effect of salinity on development time were restricted to salinities ≤30%, as *A. gambiae* and *A. coluzzii* colonies did not survive at higher salinities. Alternative analyses of the whole data set in which the missing *A. gambiae* and *A. coluzzii* data were imputed (as 100% mortality) at lethal saline concentrations yielded similar results (data not shown). Full models included the main effects salinity (CONC) and species (SPP) – with colony (COL) nested in SPP – and their interactions. The minimum adequate model was identified by analysis of deviance (ANODEV) following Crawley (Crawley, 2009), testing stepwise removal of explanatory variables and their interactions starting from the maximal model (supplementary material Table S1). The minimum adequate model was that which contained all statistically significant terms, as assessed by a significant increase in deviance indicated by likelihood ratio tests. Statistical differences between individual species pairs were assessed using *post hoc* tests, adjusting significance thresholds by Bonferroni correction.

RESULTS

Salinity tolerance was assessed through acute and chronic bioassays performed using six laboratory colonies of *A. gambiae*, *A. coluzzii* and *A. merus* (Table 1).

Salinity tolerance in acute exposure bioassays

Regardless of species, no L4 larvae survived a 24 h exposure to salinities above 50% (Fig. 1, Table 2). However, the dose–mortality response to acute saline exposure differed significantly among species (GLM: F=19.23, d.f.=2,143, P<<0.001; supplementary material Table S1). Unsurprisingly, *A. merus* had greater salinity tolerance than both obligate freshwater species (Table 3). Salinity tolerance was indistinguishable between obligate freshwater species (Table 3). Notably, the level of acute salinity tolerance was lower than expected for *A. merus* MAF (Table 2), given that this colony is routinely cultured in 50% saline (15.85 g $NaCl l^{-1}$). However, in the acute bioassays, all larvae were hatched and reared to L4 in deionized water before transfer to saline. Under these conditions, less than 25% of the L4 *A. merus* MAF larvae survived the transfer from deionized water to 50% salinity after 24 h, suggesting that saltwater tolerance is strongly influenced by the developmental stage at which exposure occurs.

Salinity tolerance in chronic exposure bioassays

The same six colonies of *A. gambiae*, *A. coluzzii* and *A. merus* were tested for salinity tolerance under a chronic exposure regime, from newly hatched larvae through to pupation (or death) (Fig. 2).

The dose–mortality responses to chronic saltwater exposure differed significantly between species (GLM: F=290.77, d.f.=2,143, P<<0.001; supplementary material Table S1). As expected, *A. merus* survived better than both obligate freshwater species, whose survivorship was indistinguishable (Table 3). For both freshwater species, survival began to decline at 20% salinity and no larvae survived salinities greater than 30% (Fig. 2, Table 4). *Anopheles merus* showed substantially higher levels of tolerance to chronic saline exposure than in the acute assays. Indeed, survivorship of the *A. merus* MAF colony (maintained since its inception in 1991 in 15.85 g $NaCl l^{-1}$) was not impacted until exposure to ~60% salinity (Fig. 2, Table 4). Interestingly, the *A. merus* OPHANSI colony, which has been maintained in deionized water for at least 260 generations, had a lower salinity tolerance than the *A. merus* MAF colony.

During the course of the chronic saltwater exposure bioassays, we noted a striking delay in the time to pupation across all three species in response to rising salinity (Fig. 3; LM: F=1947.76, d.f.=1,13154, P<<0.001; supplementary material Table S1). However, the rate and magnitude of the developmental delay was much greater for either freshwater species compared with *A. merus* (Fig. 3, Table 3). The extended time to pupation of *A. gambiae* in even the lowest salinities tested suggests that *A. merus* may have a competitive edge in nearly any natural brackish water source.

Developmental plasticity of saltwater tolerance of *A. merus*

Cohorts of our most saltwater-tolerant colony, *A. merus* MAF, exhibited relatively low salinity tolerance when exposed for the first time at the L4 stage, having been reared from hatchlings in deionized water until the initiation of the acute bioassays. This observation prompted assessment of the effect of developmental timing on saltwater tolerance in *A. merus*. To examine this question, we hatched *A. merus* MAF and OPHANSI larvae in deionized water

Table 3. Statistical tests of pairwise species differences in the dose–response to salinity

Response variable (biassay)	Comparison	F	d.f.	P-value
Mortality (acute, 24 h)	*A. merus–A. coluzzii*	30.65	1,95	<<0.001
	A. merus–A. gambiae	24.86	1,95	<<0.001
	A. gambiae–A. coluzzii	0.35	1,95	0.556
Mortality (chronic)	*A. merus–A. coluzzii*	633.19	1,95	<<0.001
	A. merus–A. gambiae	361.59	1,95	<<0.001
	A. gambiae–A. coluzzii	0.05	1,95	0.826
Delayed pupation (chronic)	*A. merus–A. coluzzii*	109.78	1,9437	<<0.001
	A. merus–A. gambiae	119.1	1,9484	<<0.001

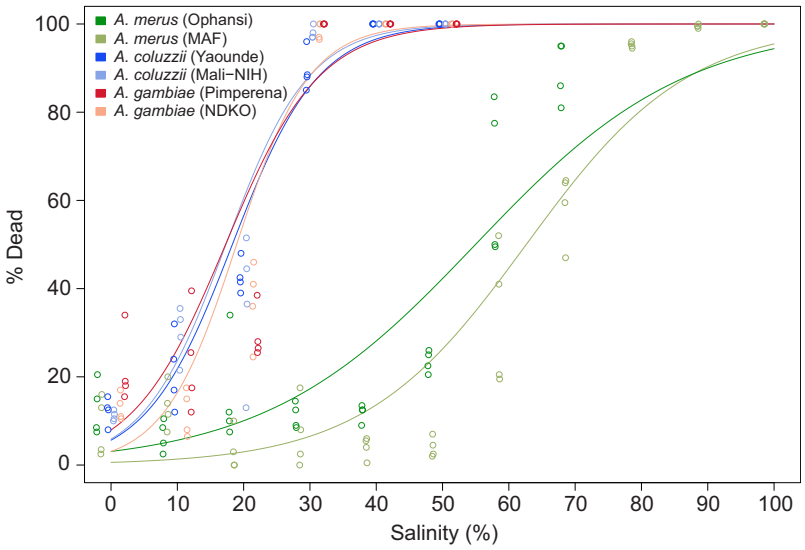

Fig. 2. Dose–mortality response to salinity measured for chronic exposure. Points denote observed mortality prior to pupation and are offset along the *x*-axis to aid readability. Logistic curves were individually fitted for each colony using all measured salinities.

and maintained them there for different lengths of time – 0, 24, 48 or 72 h – before transferring them to 50% saline solution to continue development until pupation (or death). We found that longer delays before exposure to 50% salinity indeed negatively and significantly impacted survival (GLM: F=199.42, d.f.=1,31, P<<0.001; Fig. 4; supplementary material Table S1), and that the effect differed between the two colonies of *A. merus* (GLM: F=31.44, d.f.=1,31, P<<0.001). In the OPHANSI colony, 77% of larvae pupated without exposure delay, but no more than 10% pupated in any of the delayed exposure assays. As little as a 24 h delay in exposure to saltwater drastically reduced the proportion of larvae successfully pupating. In the *A. merus* MAF colony, a 24 h exposure delay decreased survival, but a substantially greater proportion of larvae successfully pupated than was observed for OPHANSI, suggesting that MAF has a larger window of developmental plasticity in its ability to respond successfully to saltwater. However, a 48 or 72 h delay in exposure to 50% saltwater lowered the pupation proportion to levels similar to those seen in OPHANSI (Fig. 4).

Inheritance of saltwater tolerance

Hybrid F1 larvae and both parent colonies were subjected to parallel chronic salinity exposure bioassays. These tests revealed that the dose–mortality response of F1 larvae from both reciprocal crosses was intermediate to that of either parent (Fig. 5); none were able to survive at 50% salinity. However, when the female parent was *A. merus*, the dose–mortality response indicated a significantly higher tolerance of salinity by F1 hybrid larvae compared with F1 hybrids whose female parent was *A. coluzzii* (*post hoc* GLM: F=11.66, d.f.=1,47, P<0.01). This suggests that maternal inheritance (X-linkage, a cytoplasmic factor or epigenetics) contributes to salinity tolerance, but both crosses indicate that additional QTL are autosomal. Of practical importance for QTL mapping experiments, we found that exposure to the discriminating dose of 50% salinity resulted in death within 24 h for all *A. coluzzii* larvae and F1 hybrids, but mortality was less than 1% among *A. merus* larvae exposed to 50% salinity in the same time frame.

Table 4. Median and 99% lethal salinity concentrations (LC; %) estimated for parental species and F1 hybrids based on chronic exposure bioassays

Species (colony)	LC_{50}	LC_{99}	Slope
A. coluzzii (Yaounde)	18.3±0.920	48.2±2.71	0.154±0.013
A. coluzzii (Mali-NIH)	17.3±1.26	46.1±3.70	0.160±0.019
A. gambiae (NDKO)	18.9±3.73	44.2±3.73	0.182±0.025
A. gambiae (Pimperena)	17.3±5.48	49.7±5.48	0.142±0.023
A. merus (OPHANSI)	55.0±3.21	128±13.3	0.063±0.010
A. merus (MAF)	62.6±10.6	118±10.5	0.082±0.014
Mali-NIH × MAF F1	27.5±1.68	71.2±5.86	0.105±0.013
MAF × Mali-NIH F1	35.0±1.54	67.6±5.04	0.141±0.020

Data are given ±s.e.m.

Physiological basis of salinity tolerance

Physiological and pharmacological evidence based on studies of the saline-tolerant *A. albimanus* supports a model for anopheline larval ion regulation in which non-DAR cells of the larval rectum actively resorb ions from primary urine in freshwater, but become activated to secrete a hyperosmotic urine in saline water by shifting localization of membrane energizing proteins such as Na^+/K^+-ATPase (Smith et al., 2008; Smith et al., 2010). To assess whether the same physiological mechanism inferred for *A. albimanus* may underlie the salinity tolerance of *A. merus*, we made qualitative comparisons of the localization of three proteins (carbonic anhydrase, Na^+/H^+-antiporter and Na^+/K^+-ATPase) in L4 larvae of *A. gambiae* and *A. merus* that were raised in either deionized water or saline.

In accord with previous results (Smith et al., 2008), the localization patterns of carbonic anhydrase and Na^+/K^+-ATPase did not change between *A. gambiae* larvae reared in deionized water or at 20% salinity, nor did the localization of Na^+/H^+-antiporter (Fig. 6A,B). However, the localization of Na^+/K^+-ATPase shifted dramatically in larvae of the MAF colony of *A. merus*, from non-DAR cells when reared in freshwater (Fig. 6C) to DAR cells when reared at 50% salinity (Fig. 6D), as observed in *A. albimanus* (Smith et al., 2008). Notably, the protein localization pattern differed somewhat in the less salt-tolerant OPHANSI colony of *A. merus*. As was the case for the *A. merus* MAF colony, Na^+/K^+-ATPase appeared to increase in the DAR cells of OPHANSI larvae raised at 50% salinity relative to those raised in freshwater, but contrary to the localization pattern of this protein in MAF (and *A. albimanus*) there was no apparent downregulation in the non-DAR cells (Fig. 6E,F).

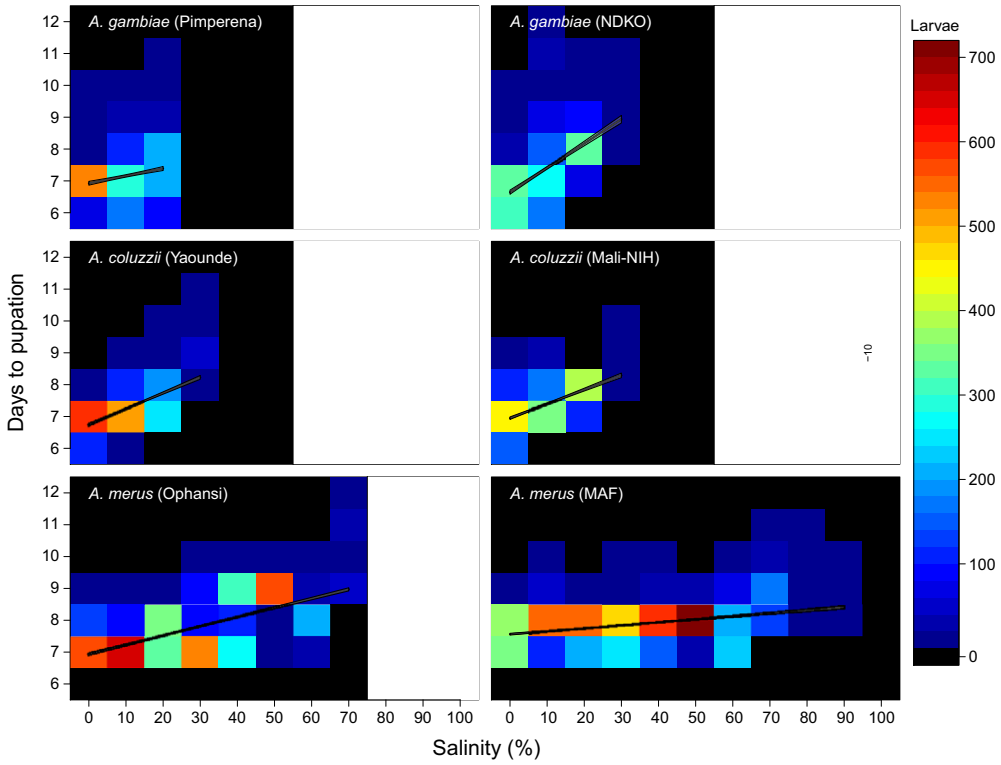

Fig. 3. Days until pupation for larvae that survived chronic exposure to different salinities. Each column represents a separate experiment, initiated with 800 larvae at a given salinity. The color of each box represents the number of larvae that pupated on that day. The white area represents salinities where a colony was not tested because of full mortality at lower salinities. Plotted lines represent the best fit obtained for each colony through linear regression; 95% confidence intervals for each fit are represented by shaped gray bands surrounding each regression line.

DISCUSSION

As far as presently known, the 95% of mosquito species obliged to live in freshwater use identical osmoregulatory mechanisms (Bradley, 1987). However, the 5% of species that are saline tolerant are dispersed across the phylogenetic tree in different mosquito subfamilies, genera and subgenera, with non-adjacent branching relationships and ancient evolutionary divergences. This suggests that saltwater tolerance has evolved repeatedly and independently during the course of mosquito diversification, since its initial radiation from a freshwater lineage in the Jurassic (Grueber and Bradley, 1994; Krzywinski et al., 2006; Reidenbach et al., 2009). Major differences in morphological and physiological mechanisms associated with saltwater tolerance are recognized between the mosquito subfamilies Anophelinae and Culicinae, which diverged at least 145 million years ago and probably over 200 million years ago (Krzywinski et al., 2006; Reidenbach et al., 2009). Although

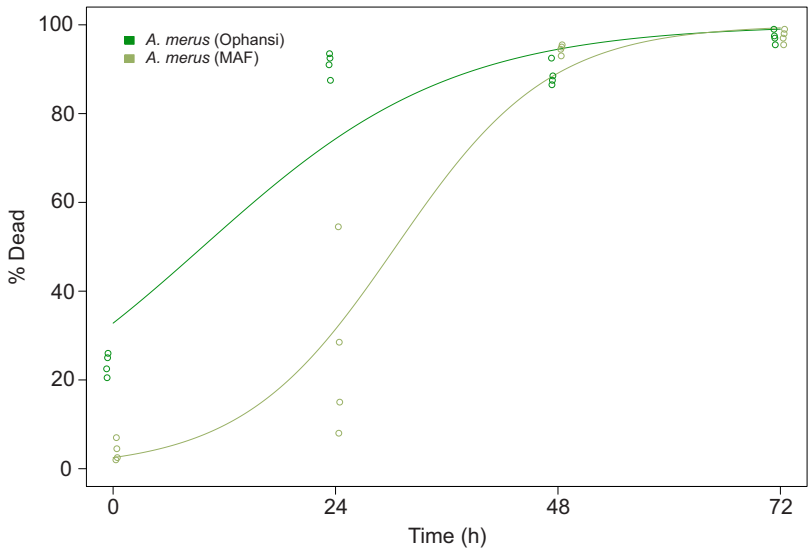

Fig. 4. The effect of developmental age on larval salinity tolerance. *Anopheles merus* larvae hatched in freshwater were transferred to saline (50%) at different developmental time points post-hatching (0, 24, 48 or 72 h) and thereafter maintained at 50% salinity until pupation (or death). Points denote observed mortality and are offset along the *x*-axis to aid readability. Logistic curves were individually fitted for each colony using all measured time points.

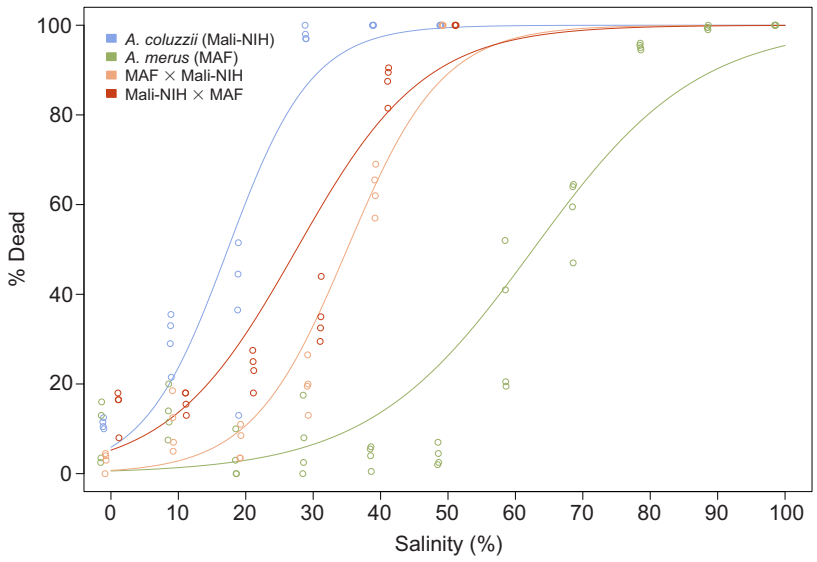

Fig. 5. Dose–mortality response to chronic saltwater exposure in larvae from parental colonies (*A. merus* MAF, *A. coluzzii* Mali-NIH) and their F1 hybrids from reciprocal crosses. For the two F1 hybrid classes, the female parent is listed first. Points denote observed mortality and are offset along the *x*-axis to aid readability. Logistic curves were individually fitted for each colony using all measured salinities.

the deep evolutionary gulf between anopheline and culicine mosquitoes is often appreciated, the depth of divergence within the genus *Anopheles* is less intuitively obvious, and the saltwater physiology of different anopheline species remains relatively unexplored compared with studies in culicines. Recent efforts to reconstruct *Anopheles* phylogeny and infer its temporal dimension suggest that subgenus *Cellia* (which contains the species studied here, *A. merus*) is a sister group to subgenus *Anopheles*, and that these sister subgenera have been separated for ~90 million years (Krzywinski et al., 2001; Krzywinski et al., 2006). The previous physiological and pharmacological characterizations of *A. albimanus* (Smith et al., 2010) refer to a species belonging to a more distantly related *Anopheles* subgenus, *Nyssorhynchus*, whose split from *Anopheles*+*Cellia* is even more ancient (e.g. >>90 million years). As such, the striking similarity in Na^+/K^+-ATPase localization patterns in the rectum between *A. albimanus* and *A. merus* was not necessarily anticipated, and although the precise mechanisms of salinity tolerance in these two species remain to be elucidated, our data suggest that there are common elements of osmoregulation that may have arisen independently, perhaps due to genetic, morphological and/or physiological constraints.

Our data suggest that the phenotypic expression of saltwater tolerance by *A. merus* larvae varies according to the developmental timing of larval exposure to salinity. There is a relatively brief developmental window that allows full expression of saltwater tolerance, but beyond this point, larvae from the same laboratory colony and even the same egg cohort are strongly curtailed in their degree of tolerance relative to those exposed without delay. Specifically, initial saline exposure within 24 h of hatching seems to allow full expression of the phenotype, while exposure after 24 h, and especially after 48 h, drastically reduces the expression of saltwater tolerance. As it appears that a major shift in the rectal localization of Na^+/K^+-ATPase is associated with salinity tolerance in both *A. merus* and *A. albimanus* (Smith et al., 2008; Smith et al., 2010), we hypothesize that whatever mechanism is responsible is induced most easily within the first 24 h after *A. merus* larval hatching. This hypothesis may account for the relatively high mortality observed in this study for *A. merus* larvae exposed to saltwater for the first time at an advanced stage (L4) of larval development, as per the design of our acute salinity bioassays. Consistent with this idea, evidence from *A. albimanus* indicated that the ability of larvae to shift rectal Na^+/K^+-ATPase was maximal at the earliest larval stage examined (2nd), and reduced at the 3rd and 4th stages (Smith et al., 2008). Whether differences in the ability to shift protein localization between species (salt tolerant and obligate freshwater) and developmental stages of the same species is due to changes in gene expression or alternative explanations is presently unknown, and a matter for future investigation.

Although the two colonies of *A. merus* used in this study are both saltwater tolerant, they differ in their degree of tolerance, potentially due to genetic changes in *A. merus* OPHANSI accompanying its long history of laboratory culture in freshwater, as distinct from MAF. In the chronic exposure bioassays, *A. merus* MAF was more saltwater tolerant than *A. merus* OPHANSI (Fig. 2). Larvae of *A. merus* OPHANSI did not survive to pupation beyond 70% salinity, and at this salinity, the few that survived took considerably longer than *A. merus* MAF to pupate (compare slopes of the regression lines in Fig. 3). By contrast, a few *A. merus* MAF larvae survived to pupation at salinities as high as 90% (Fig. 3). In the experiments where initial larval exposure to saline was delayed by 24–72 h post-hatching, *A. merus* OPHANSI showed a much-reduced ability to accommodate any delay relative to *A. merus* MAF (Fig. 4). These differences in dose–response between *A. merus* OPHANSI and MAF are associated with corresponding differences in the Na^+/K^+-ATPase localization patterns in the rectum between the two colonies. Both colonies showed localization of Na^+/K^+-ATPase in DAR cells when reared in saltwater, but only in the case of *A. merus* MAF was this event accompanied by an apparent reduction in localization of this protein in the non-DAR cells; no discernible reduction was noted in the non-DAR cells of *A. merus* OPHANSI. Although the link between the mortality data and the immunolocalization data remains to be elucidated mechanistically, the correlation of these data strongly suggests that Na^+/K^+-ATPase localization patterns in the DAR and non-DAR cells are a crucial component of saltwater tolerance, and that unknown factors regulating their localization may be key.

The salinity tolerance of *A. merus* has been noted at least since 1936 (Gebert, 1936; Mackay, 1938). While others have studied the effects of salinity on survival in this species (Mosha and Mutero, 1982; Coetzee and Le Sueur, 1988), this is the first study to fully model and compare the dose–mortality response to salinity among *A. merus*, its freshwater siblings *A. gambiae*, *A. coluzzii*

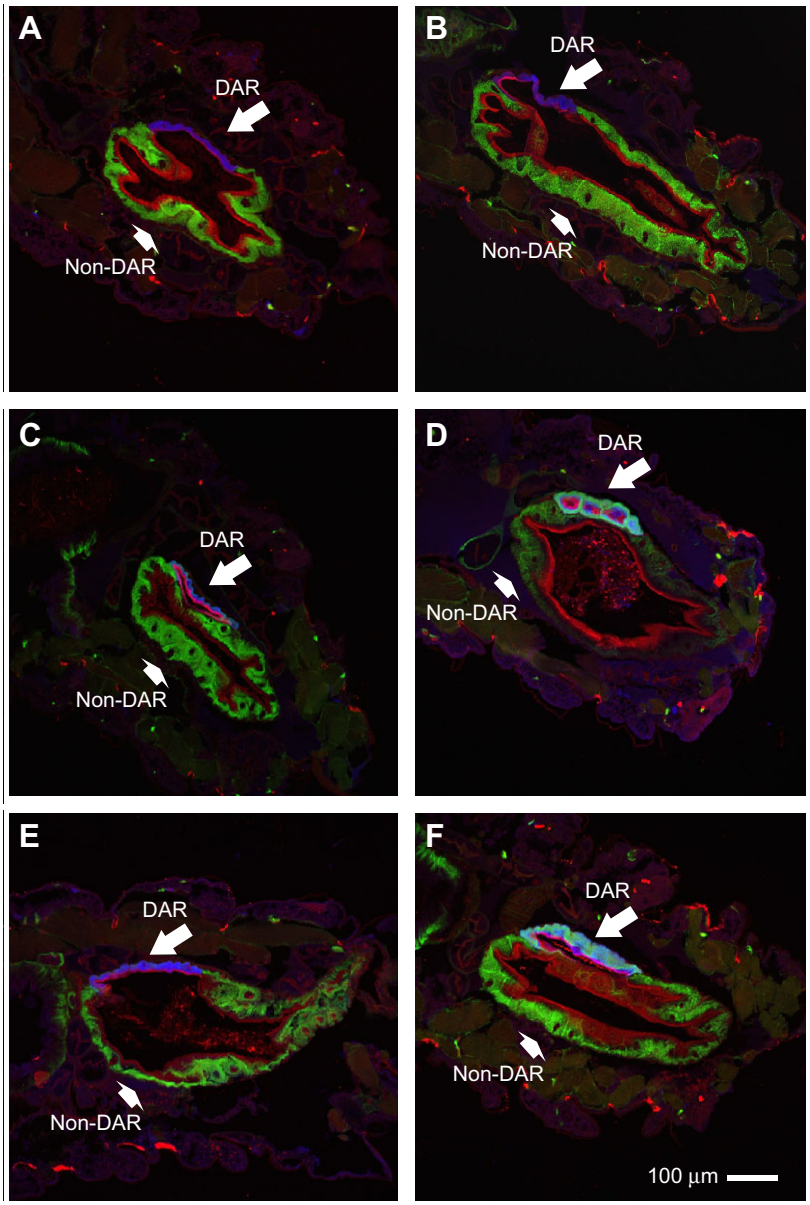

Fig. 6. Comparison of rectal localization patterns of major ion transport proteins in *A. coluzzii* and *A. merus* larvae reared in freshwater *versus* saltwater. Localization of carbonic anhydrase (CA9, blue) is indicative of DAR cells (dorsal anterior rectum) in the rectum (indicated by a long arrow). No changes in the localization of CA9 or the Na^+/H^+-antiporter (NHA-1, red), expressed along the luminal border of the rectum, were observed regardless of species or salinity. However, the localization of Na^+/K^+-ATPase (green) differed between species (and colonies) in response to salinity. In all freshwater-reared larvae [(A) *A. coluzzii* Mali-NIH, (C) *A. merus* MAF, (E) *A. merus* Ophansi], Na^+/K^+-ATPase localized only to non-DAR cells (short arrow in each panel). For *A. coluzzii* Mali-NIH larvae reared at 20% salinity (the maximum possible sublethal saline dose for this species), Na^+/K^+-ATPase localization did not change from the freshwater pattern (B). However, when *A. merus* MAF and OPHANSI larvae were reared at 50% salinity, co-localization of Na^+/K^+-ATPase with CA9 in the DAR cells, as indicated by pale blue highlighting (the merge of green and blue signals), became evident (D,F). Only in the case of *A. merus* MAF was localization of Na^+/K^+-ATPase in the DAR cells accompanied by an apparent reduction in localization of this protein in the non-DAR cells (D). The scale bar in F represents 100 µm, and all micrographs are presented at the same magnification.

and F1 *A. coluzzii–A. merus* hybrids using a defined chemical (NaCl). Importantly, our data suggest that QTL mapping of the saltwater tolerance trait in *A. merus* is tractable. At 50% salinity, there is a discrete difference in survival between the obligate freshwater species *A. coluzzii* and its sibling species *A. merus* – all *A. coluzzii* die within 24 h of exposure, and *A. merus* survive at levels indistinguishable from those of cohorts reared at lowered salinities. The fact that 50% salinity also kills all *A. coluzzii–A. merus* F1 hybrids within 24 h of exposure, taken together with their intermediate tolerance phenotype (Fig. 5), implies that survival at 50% salinity requires both *A. merus* alleles at multiple loci. In an F1 backcross to *A. merus*, we expect that backcross progeny exposed to 50% salinity without the requisite complement of homozygous *A. merus* alleles will die within 24 h, while those surviving beyond this point carry the homozygous genotypes necessary for salt tolerance, as this is a non-leaky phenotype. Accordingly, this study has laid the foundation to identify the specific genetic and physiological mechanisms underlying saltwater tolerance.

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AUTHOR CONTRIBUTIONS

B.J.W. designed and performed the bioassays together with P.N.K. P.N.K, L.V.E. and P.J.L. performed and interpreted the immunohistochemistry experiments. D.A.T. performed statistical analysis. B.J.W. and N.J.B. interpreted the results and wrote the paper, with subsequent editorial contributions and approval by all authors.

COMPETING INTERESTS

No competing interests declared.

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498 Table S1. Analysis of Deviance for main effects and their interaction for influence of salinity concentration on survival or
499 development time (to pupation) in alternative larval bioassays. In each case, the full model includes all main effects, 2nd-
500 order and 3rd-order interactions. Colonies of *An. gambiae*, *An. coluzzii* and *An. merus* are nested within species
501 (SPP/COL). Colons denote interactions.

Assay	Model Terms	Residual Deviance	d.f.	F	P
Acute Salinity	Full: CONC*SPP/COL	15.882	135		
	–CONC:SPP:COL	17.967	138	2.373	0.073
	– CONC:SPP:COL – CONC:SPP	17.969	140	1.426	0.219
	–CONC	27.542	142	5.689	<0.0001*
Chronic Salinity	Full: CONC*SPP/COL	12.603	135		
		13.678	138	3.925	0.01*
		28.179	140	34.123	<0.0001
Development time	Full: CONC*SPP/COL	5876.2	13154		
	–CONC:SPP:COL	6183	13157	228.9	<0.0001*
Exposure Delay	DELAY*COL	2.339	28		
	– DELAY:COL	2.790	29	5.188	0.031*

502 CONC, salinity concentration; SPP, species; COL, colony.