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Effect of salinity on the behavior of *Aedes aegypti* populations from the coast and plateau of southeastern Brazil

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ABSTRACT

Background & objectives: Mosquito larvae can develop in fresh, salty and brackish water. The larvae of *Aedes aegypti* develop in fresh water. However, in laboratory studies, tolerance of this species for oviposition and hatching in brackish water was observed. Immature forms of *Ae. aegypti* have also been found developing in brackish water in coastal areas. The purpose of the study was to compare the effects of salt on the biological cycle of *Ae. aegypti* populations from coastal and plateau areas of southeastern Brazil.

Methods: Aedes aegypti were collected from plateau (Taubaté) and coastal (São Sebastião) municipalities to establish colonies. Specimens of the F_1 generation were exposed to five salt concentrations (3.5, 7, 10.5, 14 and 17.5‰) to assess the oviposition and cycle development from egg hatching to adult eclosion. Deionized water was used in the control groups.

Results: Both Taubaté and São Sebastião populations oviposited in all salt concentrations tested; however, development occurred in all except in the 17.5% concentration. Significant differences in development and adult size were observed at intermediate concentrations (São Sebastião from 10.5‰ and Taubaté from 7‰, for both variables) between the two populations.

Interpretation & conclusion: The results of this study showed different response patterns to salinity between the two populations, suggesting better adaptive adjustment of the coastal population to the metabolic constraints exerted by salt. The implications of this adaptation are discussed, and additional studies are suggested to evaluate the mechanisms that determinate the adaptive processes of *Ae. aegypti* in brackish water and the implications to its vectorial capacity.

Key words Adaptation; *Aedes aegypti*; arbovirus; biological cycle; breeding sites; salinity

INTRODUCTION

Aedes aegypti (Diptera: Culicidae) is originally from wild ecotype, where it is found breeding in water rich in organic matter that accumulates in treeholes, bamboo internodes, leaves and the peels of fallen fruits, puddles on the ground, and rock pools. The spread of this mosquito to other tropical and subtropical regions between the latitudes 35° N and 35° S occurred due to its successful colonization in the urban environment, with the immature forms developing in artificial containers with relatively clean water. Plant pots, tyres and disposable containers were the most common breeding sites in the beginning of the colonization in the urban environment[1–2], where the presence of predators and competitors was rare than in natural breeding sites[3].

Given these circumstances, the management and elimination of breeding sites, taking local characteristics into account, were considered as the main strategies of vector control programs. Factors that can contribute to different control measures being adopted in different locations include: local differences in the duration of infestations, patterns of landuse and occupation, climate regimes, development and establishment of production and trade processes and infrastructure, public education policies, environmental sanitation and entomological surveillance and control[4–5].

The intensification of larval control activities in municipalities affected by dengue and urban yellow fever seems to cause changes in the behavior of selection of oviposition containers by *Ae. aegypti*. This factor has been suggested as responsible for the increased presence of the mosquito in difficult-to-access urban breeding sites, such as water tanks, slabs and gutters, along with containers with "dirty" water, such as drains, rain channels, treeholes and ornamental bromeliads, which illustrate the wide range of water physicochemical conditions in these microhabitats[1].

The larvae of several mosquito species are tolerant to salinity, including some species of the genus *Aedes*[6]. Observations related to *Ae. aegypti* have indicated that the species occurs in salt concentrations of up to 16‰, mainly in abandoned vessels[7] and, especially in Brazil, in vessels still in use but stored in marinas[8]. In the laboratory, larvae of this species exposed to different salt concentrations are able to survive until a maximum concentration of 14‰ is reached and the adults that emerge are smaller than the average size of adults that emerge in fresh water[9].

The Culicidae can be classified into three different groups according to their osmoregulation strategy: (a) freshwater osmoregulators; (b) euryhaline osmoregulators—have specific structures adapted for osmoregulation at different salt concentration gradients; and (c) euryhaline osmoconformists—tolerant to salinity exposure due to their acclimation capacity[6].

The study of the osmoconformist behavior in coastal populations of *Ae. aegypti* and in populations that do or do not maintain gene flow with the coastal populations is necessary to assist in decision making and to define control measures specific to populations of different regions. The aim of this study was to evaluate and compare the biological cycles of *Ae. aegypti* populations regarding tolerance to salinity.

MATERIAL & METHODS

Ae. aegypti populations: Egg collection and colony maintenance

Two *Ae. aegypti* populations from two distinct municipalities, São Sebastião and Taubaté, São Paulo state, Brazil, were used in this study. São Sebastião (23°47'37" S: 45°24'44" W) is located on the north coast of São Paulo and is an urban area at an altitude of 102 m above the mean sea level. Taubaté (23°01'08" S: 45°33'23" W) is located in the plateau region of the State of São Paulo, in the Paraíba Valley, 65 km from the coast at an altitude from 548 to 671 m above the mean sea level. The municipalities are separated from each other by the Serra do Mar mountains, which comprise an orographic barrier, with an altitude range of 800 to 1,200 m above the mean sea level. São Sebastião and Taubaté are located 209 km and 130 km, respectively, from the state capital, the city of São Paulo.

For the establishment of F_0 generation colonies, 50 ovitraps were placed in urban areas of the two municipalities under study. Eight collections with 5-days of exposure were performed from 7 September 2010 to 30 October 2010. In the municipality of São Sebastião, the ovitraps were placed in areas of occurrence of immature larvae in brackish water, near the coast. In Taubaté, the ovitraps were placed in an older infestation area.

In the insectary environment, the climatic conditions were 28°C ± 1°C with 75 ± 5% relative humidity and a photoperiod of 12 L:12 D hours (from 0600 to 1800 hrs)[10].

The eggs were counted and submerged in dechlorinated water in white plastic trays. Tropical fish feed was added at the proportion of 1 egg/2 ml of water. After hatching, the larvae were fed until pupation[11], and the pupae were placed in separate smaller containers until adult eclosion occurred.

An F_0 generation cage was assembled for the population from each municipality by individually transferring 100 adult females and 50 adult males to the cage. These insects were fed with 10% honey solution and apple.

After 3-days of emergence, the females were allowed to feed on blood meal from Swiss mice for 30 min on alternate days, from monday to friday, for three weeks. For this procedure, the mice were anesthetized according to protocols for the use of animals in scientific experiments. The mice were rotated so that the two mice, one per cage, could rest for 10 consecutive days after being used; therefore, eight mice were used during the experiment.

To obtain the F_1 generation, an amber glass container covered internally with filter paper and filled to 1/3 of its capacity with water decanted for 24 h was used as the oviposition substrate in each cage.

Effect of salt on the biological cycle

The eggs of F_1 were used to raise the experimental individuals. The eggs were immersed in salty water at a proportion of 1 egg/2 ml of salt water in white trays. The salt concentrations tested were 3.5, 7, 10.5, 14 and 17.5‰. Deionized water was used in the control group[9]. The time of hatching after immersing the eggs in both experimental and control units was 24 h. The L_1 larvae were transferred to 230 ml glass containers at a proportion of 1.67 ml/larva, thereby setting a depth of 1.5 cm. In all concentrations tested, 30 larvae were put in each experimental unit.

The salt solutions consisted of sea salt dissolved in deionized water. The salt concentrations were based on the concentration ranges previously tested by Clarck *et al*[9] and the maximum salt concentration measured in the field in the studied area[8]. The salinity was measured using a Multiparameter Meter Sension™ 156 portable device. The experiment was replicated thrice, with a total of 1080 larvae. The food, tropical fish feed[11], was provided daily to the larvae after an exchange of 2/3rd of the water volume. This procedure was followed until pupation. Salinity, pH and water temperature were measured daily. Dead larvae were removed in the morning between 0900 and 1000 hrs and in the afternoon between 1600 and 1700 hrs.

The adults were killed with ethyl acetate 24 h post-emergence. To assess possible changes in wing length, the left wings of males and females were dissected. Each wing was measured with the aid of a binocular microscope, from the alular notch to the distal wing margin, excluding the fringe scales[12]. The wing size was determined by the mean length for each concentration and sex.

Effect of salt on oviposition behavior

Two colonies were established using the same methods described in the previous experiment. To evaluate the effect of salt on oviposition behavior, *Ae. aegypti* females were exposed to oviposition containers covered with filter paper, each containing a different salt concentration (3.5, 7, 10.5, 14 and 17.5‰), and the control group was exposed to deionized water. The eggs were collected and the filter paper with the respective solutions was replaced daily. When the filter paper was replaced, the oviposition containers were positioned in a different place inside the cage, following the clockwise direction. This procedure was performed for 30 consecutive days.

Data analysis

To evaluate the effects of salinity on larval survival, the number of adults that emerged after exposure to each salt concentration and the control were compared. For the analysis of development time, the number of days elapsed from larval hatching to adult emergence were compared. The adult size was estimated from the wing length, and oviposition was estimated by the total number of eggs laid at each concentration tested.

The survival at each concentration tested was determined by the mean percentage of survivors. Analysis of development time, adult size and oviposition, was done using the Kruskal-Wallis (KW) test, followed by the Dwass-Steel-Chritchlow-Flingner test for multiple comparisons (alpha = 0.05). The programs Statistica (StatSoft, Inc. 2013, version 12. Tulsa, OK, USA) and StatsDirect (StatsDirect Ltd 2012, version 2.7.9. Cheshire, WA, UK) were used for the statistical analysis.

Maintenance and care of the experimental animals followed the guidelines for use of laboratory animals in research established by the Ethics Committee on Animal Experimentation of the University of Taubaté (Universidade de Taubaté–UNITAU), which approved the research protocol (Record No.: CEEA/UNITAU-006/2009).

RESULTS

Effect of salt on the biological cycle

Survival: The survival curves of both populations were similar along the salinity gradient tested. Both populations (Taubaté and São Sebastião) had survival rates of > 90% in the control concentration and close to 100% at the lowest concentration tested (3.5‰). A sharp decline of approximately 50% in the survival rate was observed at the 14‰ concentration, and total mortality occurred at the 17.5‰ concentration (Fig. 1).

Development time

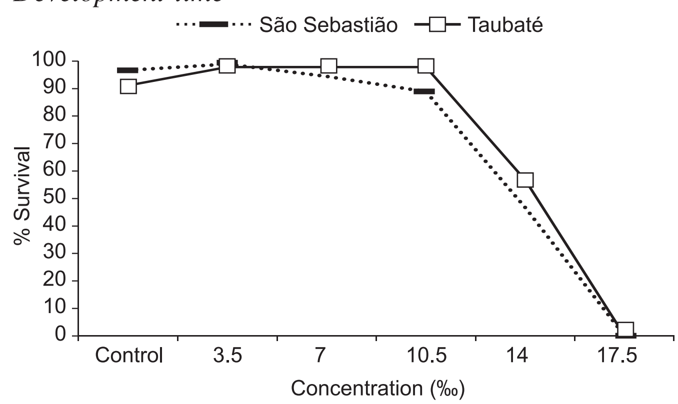

Fig. 1: Effect of salinity on survival (n=3 replicates/population). Each replicate consisted of 30 larvae/concentration totaling 360 larvae.

The number of days elapsed from larval hatching to adult emergence was significantly different when the concentrations were compared within the same population (São Sebastião: KW 148, $p = 0.0$; Taubaté: KW 191, $p = 0.0$).

There were associations between development time and salinity gradient for both males and females of the two populations. For the São Sebastião population, the differences in development time were significant starting at the 10.5‰ concentration for both males and the females (Males: KW 94, $p < 0.0$; Females: KW 60, $p < 0.0$). For the Taubaté population, the differences were significant starting at the 7‰ concentration for both males and the females (Males: KW 100, $p < 0.0$; Females: KW 98, $p < 0.0$) (Fig. 2).

When the development times of males and females were compared within the same concentration, the times were similar at the 10, 5 and 14‰ concentrations for the São Sebastião population (São Sebastião: KW 190, $p < 0.0$;

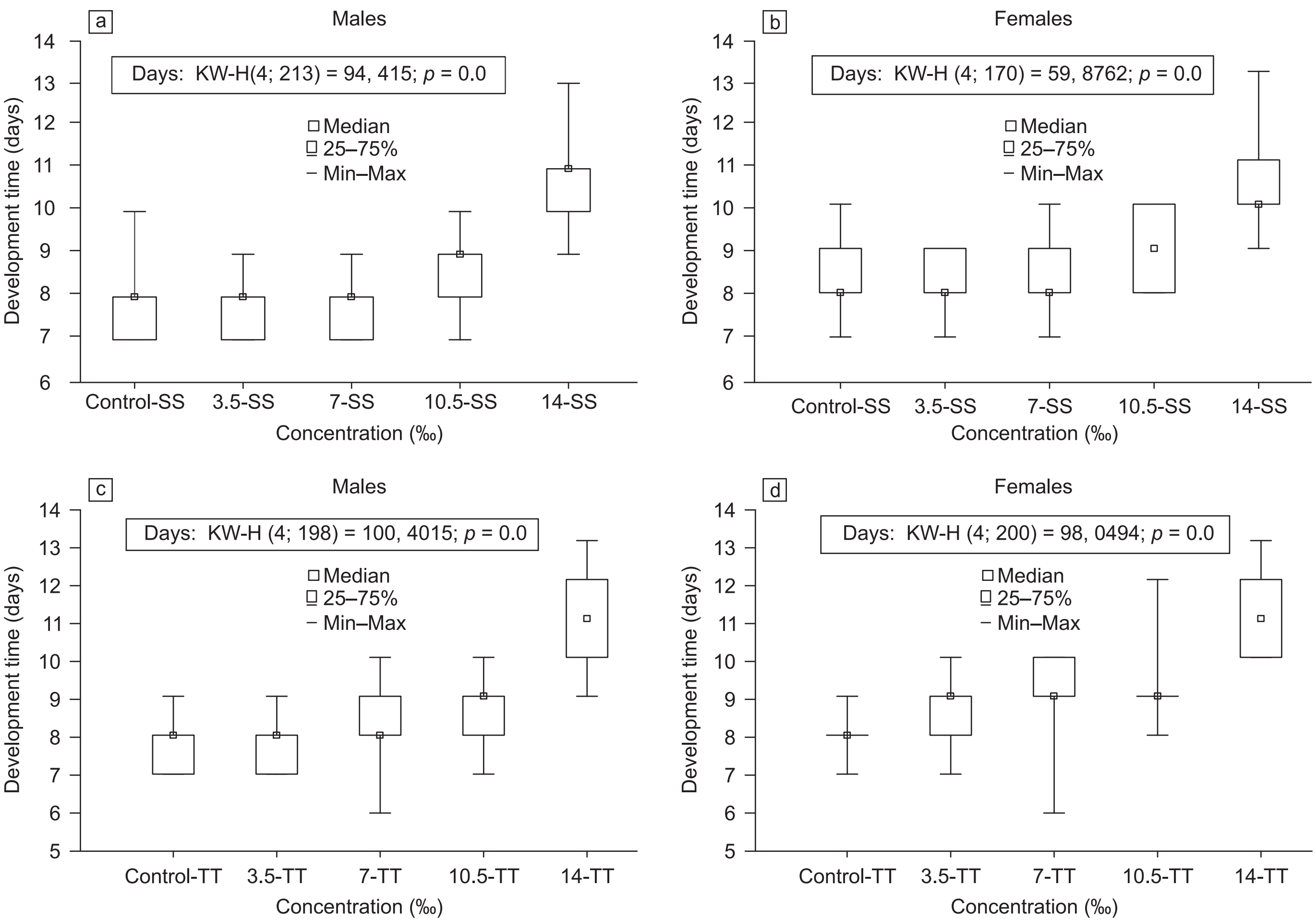

Fig. 2: Development times (in days) of males and females at each concentration for each population: São Sebastião [(a) Males; (b) Females], and Taubaté [(c) Males; (d) Females].

Taubaté: KW 220, p<0.0) and at the 14% concentration for the Taubaté population .

Comparison of development times within each sex per population indicated significant differences for females at the 7‰ concentration (Males: KW 198, p<0.0; Females: KW 163, p<0.0) (Fig. 3).

Adult size

Adult size was estimated from the mean length of the left wing. The salinity affected the adult sizes of both populations studied. The growth rates decreased significantly with increased salinity. The mean wing lengths were different among concentrations within the same population (São Sebastião: KW 23, p=0.0; Taubaté: KW 67, p=0.0). The differences were significant at the 10.5 and 14‰ concentrations for both populations and at the 7‰ concentration for the Taubaté population.

When the mean wing lengths of males and females were compared among concentrations within each population, significant differences were observed starting at the 10.5‰ concentration for the São Sebastião population (Males: KW 39, p<0.0; Females: KW 54, p<0.0). For the Taubaté population, significant differences were observed along the entire salinity gradient tested (Males: KW 105, p<0.0; Females: KW 106, p<0.0). These differences were observed for both sexes and populations (Fig. 4).

When mean wing lengths were compared between the sexes at the same concentration within the same population, significant differences were observed at all concentrations and for both populations (São Sebastião: KW 287, p<0.0; Taubaté: KW 323, p<0.0). When comparing the mean wing lengths of the same sex between the populations, significant differences were observed between males and the control group (Males: KW 152, p<0.0; Females: KW 163, p<0.0) (Fig. 5).

Effect of salt on oviposition behavior

When the oviposition behavior was compared among concentrations within the same population, there were

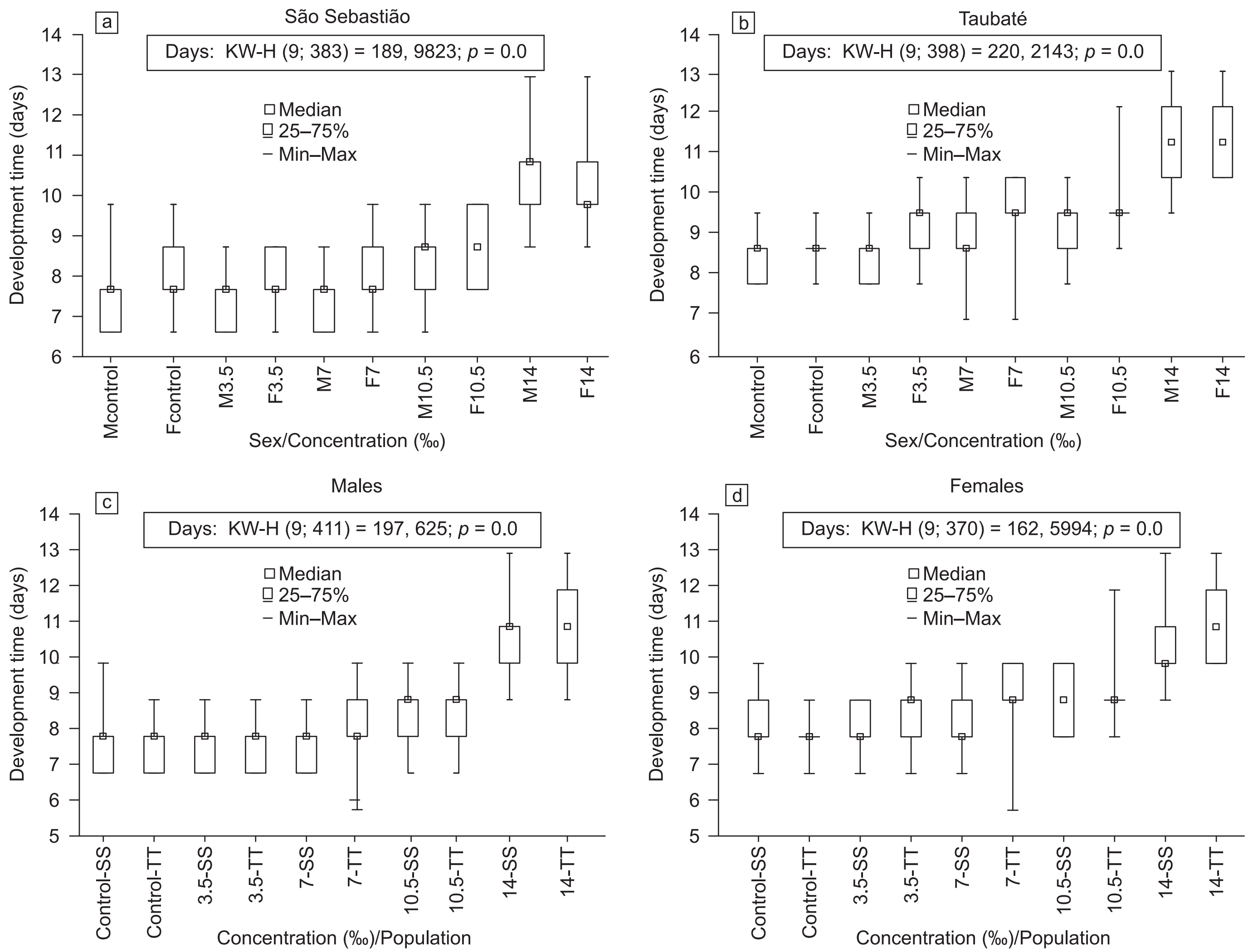

Fig. 3: Development times (in days) of males and females at each concentration per population, São Sebastião (a); and Taubaté (b). Development times (in days) of males (c); and females (d) of the two populations.

similarities in the numbers of eggs laid: KW 10.7, *p*=0.06; and KW 1.6, *p*=0.9 for São Sebastião and Taubaté, respectively. The comparison of the total number of eggs laid over all salinity concentrations between the populations showed the same similarity (KW 18.8; *p*=0.06).

DISCUSSION

Aedes aegypti responses to the salinity gradient tested in this study were similar in both populations, corroborating with the hypothesis that the euryhaline osmoconformist behavior is an intrinsic characteristic of the species[6]. However, significant differences were observed in the responses to the intermediate concentrations between the two populations. This finding indicates a greater tolerance of the São Sebastião population to salinity compared to the Taubaté population. In addition, the evaluation of the confidence intervals of the variables tested revealed that the São Sebastião specimens had less variation in their response to each treatment compared to the Taubaté specimens. Despite the need for additional studies with larger numbers of locations to assess such differences, these results suggest that salinity exerted some degree of selective pressure on the coastal population, conferring greater specialization due to this environmental condition.

Some authors discuss this adaptive process in mosquito populations from areas influenced by salt in both coastal[8,13] and continental regions[7, 14]. In the present study, we compared two *Ae. aegypti* populations, with and without coastal environment influence, which we considered functionally separated regarding gene flow[15]. The separation occurs due to an important orographic barrier with a mean altitude of 700 m above sea level[16].

Regarding oviposition, the females of the populations oviposited at all salt concentrations offered, including the

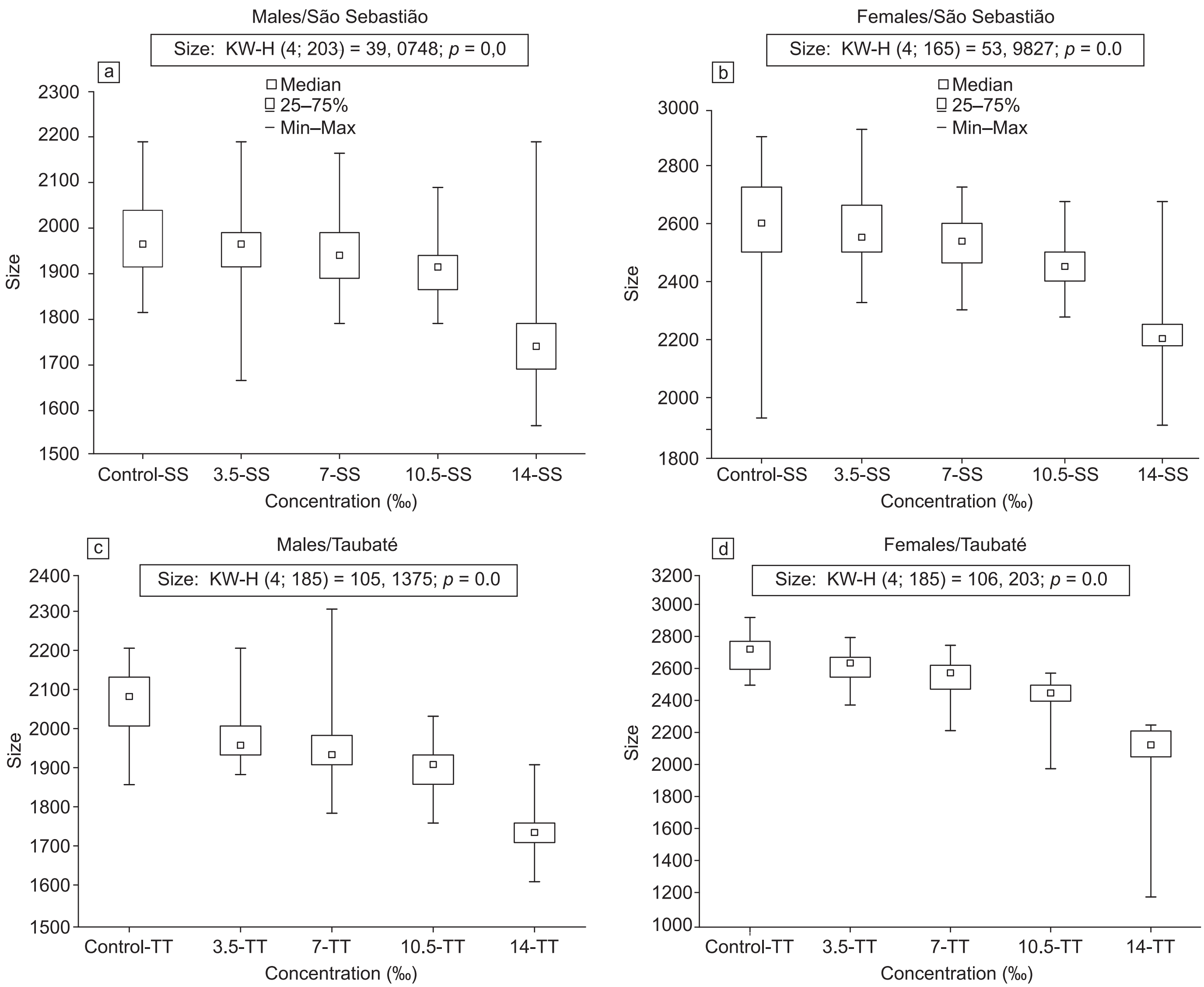

Fig. 4: Wing lengths of males and females at each concentration within each population. São Sebastião [Males (a); Females (b)] and Taubaté [Males (c); Females (d)].

17‰ concentration, in which no survival of immature larvae was recorded.

Container selection is critical for the survival and population dynamics of mosquitos[17] and has important implications in species control. In an oviposition experiment performed in the field, ovitraps with salt gradients ranging from 2 to 20‰ were used, and *Ae. aegypti* eggs were found in traps with salt concentrations of up to 18‰[13]. In a study conducted in the laboratory, oviposition was observed at concentrations of up to 20‰[18]. Under natural conditions, larvae and pupae were found in various container types with salinities between 0.1 and 13.5‰ in São Sebastião[8] and between 2 and 15‰ in Sri Lanka[7].

In addition to oviposition at all concentrations tested, there were no significant differences in the number of eggs laid among the concentrations within the same population and in the number of eggs laid at the same concentration between the two populations. Some substances used by humans or naturally found in the water of potential breeding sites may exert a repellent effect on gravid females, inhibition of oviposition and repellence[19]. Our results showed that salt neither caused a repellent effect nor inhibited oviposition. In contrast, in a study conducted with specimens from Maceió, northeastern Brazil, a decline was observed in the number of eggs laid starting at a concentration of 10.1‰ NaCl compared to distilled water and at the 1.8 and 5‰ concentrations[18]. Such conflicting results should be investigated in future studies, considering the different experimental designs used in our study and the study performed in Maceió. *Ae. aegypti* seems to find containers with low salt concentrations favorable to oviposition, as previously reported at the con-

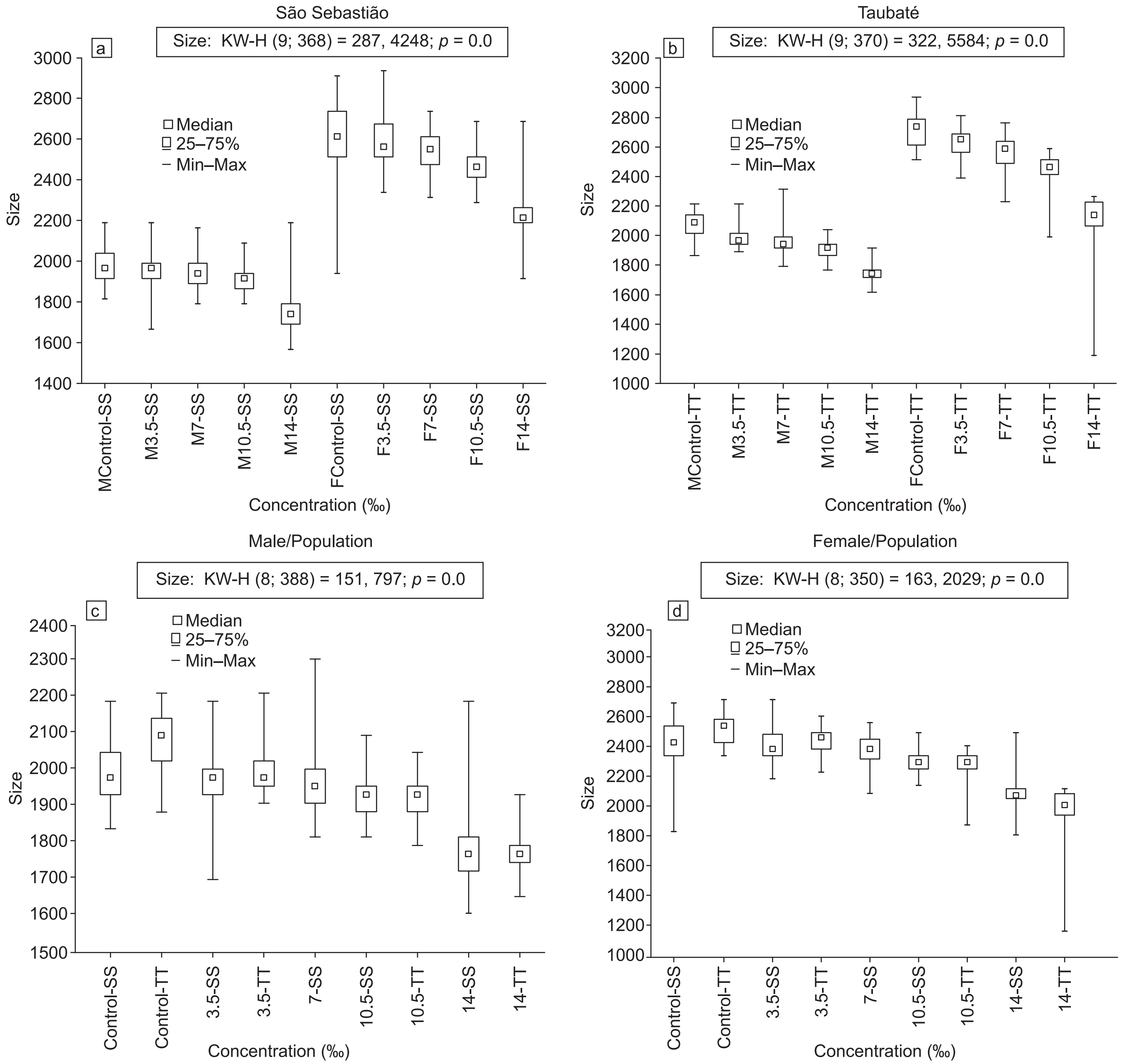

Fig. 5: Wing lengths of males and females at different concentrations per population: São Sebastião (a) and Taubaté (b). Wing lengths of males (c) and females (d) at each concentration between the two populations.

centration of 2.5‰[19]. Under adverse conditions, mosquito larvae may have difficulties in assimilating nutrients and may spend more energy to maintain their regular and vital physiological processes, resulting in losses in cycle finalization and adult emergence[9].

In laboratory tests, it has been observed that *Ae. aegypti* survival in deionized water, used as a control, was lower than survival at a 3.5‰ salt concentration[9]. In the present study, a similar situation was observed for the Taubaté population, which displayed slightly higher survival at the initial concentrations in relation to the control group compared to the São Sebastião population.

Overall, the survival curves of the two populations evaluated were quite similar. A sharp drop in the survival rate to approximately 50% occurred at the 14‰ concentration. Similar survival values were reported in other laboratory studies, despite the fact that these studies had different experimental designs, in which the larvae hatched in fresh water were transferred directly to bottles with different salt concentrations. This procedure may have caused a physiological or metabolic shock in the specimens, as mentioned by the authors[9, 13, 20].

Regarding the total mortality recorded at the 17.5‰ concentration, several studies performed in the field have reported finding live larvae and pupae in containers with brackish water with a maximum concentration of 16‰[7, 8, 13, 20].

The increase in the development time under natural conditions may have deleterious effects on the survival of the specimens due to longer exposure to adversities, including competition, predation, the presence of toxic substances and weather extremes[21]. Likewise, adult size can be affected by these determinants and, particularly in relation to the size of females, can result in reduced reproductive success and vectorial capacity[22].

The positive relationship between salt concentration and development time observed in both populations tested in the present study was also observed in populations from other locations[9]. The comparison of the development time of each sex between the populations studied indicated that better responses to salinity are found in both males and females of the São Sebastião population. Furthermore, salinity did not affect the natural differences between the sexes regarding their larval development times[14] because the concentrations at which increased development time was observed were the same for both sexes of each population.

The increase in the development time observed starting at the 7‰ concentration for the Taubaté specimens compared to the concentration of 10.5‰ for the São Sebastião specimens suggests that the latter has a distinct response to salinity and is less affected during ontogenesis. The lower variability in development time among the São Sebastião specimens suggests that this response to salinity results from a consolidated adaptive process, offering greater ecological valence in the coastal zone. However, the question remains as to whether this characteristic results from the adaptive pressure exerted by salinity on breeding sites *in loco* or from the fact that the *Ae. aegypti* variant that colonized the coastal area contained significant representation of this phenotype in its initial genetic population structure.

It is important to note that the effects of salinity on development time at the 10.5 and 14‰ concentrations for both populations and at the 7‰ concentration for the Taubaté population can be confirmed by the decreased wing sizes. This decrease, caused by increases in the salinity concentration, corroborates previous studies[9, 20], and the salinity effect is more evident for wing size than development time.

For the São Sebastião specimens, the decreases in the wing lengths of males and females observed starting at the 10.5‰ concentration suggest greater tolerance to salinity compared to the Taubaté specimens, in which differences in the wing sizes of both sexes were observed starting at the lowest concentration tested. Such differences may also characterize a better adaptive adjustment to metabolic constraints exerted by salt in the coastal population.

It is important to emphasize the differences found in the present study regarding the smaller size of adult males in the control group of the São Sebastião population compared to the control group of the Taubaté population. Wings of males and females assume peculiar functions in each sex and therefore, do not follow the same development path[23]. In this organ, development is sex-specific[24], which agrees with the higher genetic plasticity suggested for the mosquitoes from São Sebastião. It is known that one of the factors that may adversely affect the vectorial capacity of a species is the adult size[22]; therefore, studies to assess the impact of the decreased size of adults of the São Sebastião population on arbovirus transmission are needed.

Despite the differences detected, the Taubaté population is able to colonize and develop in breeding sites with the same salt concentrations as the São Sebastião population. The performance of control measures directed to a likely specialization of the species to life in saline environments might be ineffective because the subpopulation may suffer constant replacement by other equally adapted subpopulations. Still, it is interesting to note that in studies of genetic structure characterization, the São Sebastião population only shares haplotypes with other coastal locations, such as Fort Lauderdale (USA), Lima (Peru) and Maracay (Venezuela), while the Taubaté population shares haplotypes with other Brazilian continental locations such as Cuiabá, Campo Grande, São José do Rio Preto and Belém[25–26].

CONCLUSION

In this study, we found that both *Ae. aegypti* populations, the coastal population and the plateau population, were able to oviposit in brackish water at the same salt concentrations of up to 17‰ and to develop in salt concentrations of up to 14‰. However, differences in development and size were observed between adults of the two municipalities, indicating better adaptive adjustment by the coastal population to the metabolic constraints exerted by salt. More accurate studies on the mechanisms that determine the adaptive processes of *Ae. aegypti* in brackish water and the possible implications on its vectorial and epidemiological capacity in coastal areas are suggested.

Conflict of interest: The authors declare no conflicts of interest.

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