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Differences in the effects of salinity on larval growth and developmental programs of a freshwater and a euryhaline mosquito species (Insecta: Diptera, Culicidae)

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Summary

The effects of salinity on growth and development of the euryhaline *Ochlerotatus taeniorhynchus* and the freshwater *Aedes aegypti* are compared. *O. taeniorhynchus* grow larger, and have greater intrinsic growth rates, than *A. aegypti*. Females of each species attain greater mass, take longer to develop, and have greater growth rates than do males. In *O. taeniorhynchus*, pupal mass, larval stage duration and growth rates (dry mass) increase with salinity, whereas growth rates (wet mass) remain constant across salinities, reflecting a decrease in percent body water. The pupal mass (wet or dry) of *O. taeniorhynchus* is determined primarily by effects of salinity on the rate of assimilation of dry mass, because the latter contributes very strongly to final pupal mass in both species. In contrast, the duration of *A. aegypti* larval stage follows a U-shaped curve, with most rapid development at intermediate salinities. Growth rates of *A. aegypti* decrease with increasing salinity, and percent body water is constant across salinities. As for *O. taeniorhynchus*, duration of *A. aegypti* larval stage increases at high salinity. However, this increase in larval stage duration cannot compensate for the decrease in growth rate at high salinity, resulting in an overall decrease in both wet and dry pupal mass at high salinity. Thus, salinity has fundamentally different effects on developmental programs and phenotypic plasticity in the two species investigated.

Key words: mosquito larvae, salinity, life history, growth rate, developmental rate, *Aedes aegypti*, *Ochlerotatus taeniorhynchus*, insect.

Introduction

Larval mosquitoes are found in a variety of aquatic habitats, including freshwater and saline environments of diverse composition. Larval mosquitoes with three distinct osmoregulatory strategies can be described: freshwater osmoregulators, euryhaline osmoregulators and euryhaline osmoconformers (Bradley, 1987). At least two of these strategies are observed in closely related genera within the tribe Aedini, *Ochlerotatus* and *Aedes* (*Ochlerotatus* was considered a subgenus of *Aedes* until recently; Reinert, 2000). The evolution of a salt-secreting rectal segment has enabled some species, including *O. taeniorhynchus* (Wiedemann), to tolerate salinities in excess of seawater (Bradley and Phillips, 1975, 1977). This ability in turn allows them to exploit highly productive habitats that are inhospitable to freshwater forms. For example, *O. taeniorhynchus* is known as a salt marsh mosquito because it inhabits estuaries and salt marshes where salinities fluctuate from essentially freshwater to greater than seawater following rainfall or evaporation of isolated pools. Obligate freshwater forms including *A. aegypti* (L.) lack this salt gland, and are thus limited in their distribution to freshwater environments (those more dilute than their normal hemolymph osmotic pressure; Bradley, 1987). *Aedes aegypti* is found primarily in small, isolated bodies of water such as tin cans and discarded tires.

While survival to adulthood has obvious fitness consequences, environmental influences on more subtle aspects of growth and development are also important in determining fitness. Mosquito larvae face a number of threats, including predation and desiccation of habitats, and mosquitoes are typically characterized by rapid completion of the life cycle. Rapid development allows multivoltine insects, including mosquitoes, to complete more generations during the breeding season and accomplish more explosive population growths during favorable periods. Among insects, females are generally larger than males, and female size is related to fecundity. There thus exists a selective trade-off between rapid development and size at maturity. Phenotypic traits such as growth rates and mass are affected by gene–environment interactions, and a number of environmental factors are known to influence mosquito growth and development. These include

salinity, temperature, density, food supplies, and physical size and shape of the larval habitat (Clark et al., 2004; Nayar, 1968, 1969; Nayar and Sauerman, 1970; Trpis and Horsfall, 1969; McGinnis and Brust, 1983).

Because so many factors influence growth rates of larval mosquitoes, comparisons of the responses of different species to changes in a particular environmental parameter must be carefully controlled. To this end, in this study larvae of the two species were reared at the same density, in the same volume of water, and in identical containers. The same feeding protocol, photoperiod and temperature regimes were also used. These factors are all known to influence growth and development. In this way, the responses of larvae of the two species to salinity, when subjected to conditions within their respective physiological capacities, could be compared directly.

The salt-secreting gland found in euryhaline species such as *O. taeniorhynchus*, lacking in freshwater forms including *A. aegypti*, is hypothesized to alter the energetics of ionoregulation sufficiently to impact growth and developmental parameters. We predicted that *A. aegypti* and *O. taeniorhynchus* would respond in a similar way to increased salt concentrations at the low end of the salinity range, due to their shared mechanisms for survival in freshwater, and that their responses would diverge as salinity increased beyond that typically experienced by freshwater larvae. Instead, what we found was that salinity has fundamentally different effects on growth and developmental programs in the two species examined.

Materials and methods

Rearing of mosquitoes

Colonies of *Aedes aegypti* L. and *Ochlerotatus taeniorhynchus* Wiedemann were established using eggs from the Florida Medical Entomology Laboratory, in Vero Beach, FL, USA. Eggs were hatched in deionized water. The next day, 20 larvae were transferred to 50 ml of the appropriate composition in Ziploc containers (236 ml total container volume; water 1 cm deep), and fed ground TetraMin flakes (TetraWerke, Melle, Germany). This protocol was repeated for a number of replicates at each salinity. Larvae were maintained on a 16 h:8 h L:D photoperiod at 26°C. The water was replaced and the larvae fed each day until death or pupation occurred. Duration of the larval stage was determined to the nearest 24 h, and pupal wet mass was determined to the nearest 10 μg using a Toledo AX205 deltarange balance (Mettler, Columbus, OH, USA). In some instances, pupae were then dried at 65°C for 24 h and reweighed to determine dry mass. Masses of dead larvae or pupae were not determined (mosquitoes have pupae that are highly mobile, and dead pupae can be readily distinguished by their failure to remain within the water column, swim to the surface to obtain air, or respond to the presence of the investigator).

Solutions

Instant Ocean artificial seawater (Aquarium Systems, Mentor, OH, USA) was used to make up rearing solutions. Larval *A. aegypti* were reared in concentrations of 0, 3.5, 7, 10.5, 14 and 17.5 g l^{-1} (encompassing the range from deionized water to approximately 50% seawater), while larval *O. taeniorhynchus* were reared in concentrations of 0, 7, 14, 21, 28 and 35 g l^{-1} (encompassing the range from deionized water to approximately full-strength seawater). The osmotic pressures of these sea salt solutions were determined to be as follows (g l^{-1}: mOsm l^{-1}) using a Wescor 5500 Vapor Pressure Osmometer (Logan, UT, USA): 0, OP not measured; 3.5, 83; 7, 167.5; 10.5, 278; 14, 356; 17.5, 442; 21, 528; 28, 695.6; 35, 897.

Statistical analyses

Effects of salinity were modeled using mixed linear models (SAS mixed procedure, SAS Institute Inc. 1997). Full models that include species, salinity, salinity squared, sex and all interactions were fitted in order to compare salinity dependence between the two species. These were followed with separate models fitted for each species, with sex, salinity, salinity squared and their interactions. This quadratic term was included to fit curvature in the relationship between life history parameters and salinity. For each of the five life history parameters, duration of the larval stage, pupal wet mass, pupal dry mass, growth rate of wet mass and growth rate of dry mass, a model containing sex, salinity and salinity squared was first fitted. Where the quadratic term was not statistically significant, it was dropped from the model and a purely linear model was fitted. Because preliminary models found no significant sex-dependent response to salinity, no such interaction terms are included in the final best-fit models. A single life history parameter, days to pupation, was log-transformed to improve normality.

Path diagrams of the relationships among salinity, growth parameters (log-transformed days to pupation and assimilation of dry mass), and final pupal dry mass were constructed using the SAS REG procedure with the STB option (Pedhazur, 1982; SAS Institute Inc. 1997). Separate diagrams were made for each sex and species, but within each species the two sexes are shown in a single diagram, since no substantial differences remained after standardizing for overall differences in larval size.

Results

Survival

Both species show approximately 80% survival in nominally deionized water, in which all ions are obtained from the food. Survival of *A. aegypti* increases to near 100% as salinity increases from 0 to 3.5 g l^{-1}, then drops precipitously as salinity increases past 10.5 g l^{-1} with only 52% surviving 14 g l^{-1} and none surviving 17.5 g l^{-1} (Fig. 1). In contrast, survival of *O. taeniorhynchus* peaks at 14 g l^{-1} and then declines more slowly, with 52% surviving in full strength seawater (35 g l^{-1}) (Fig. 1).

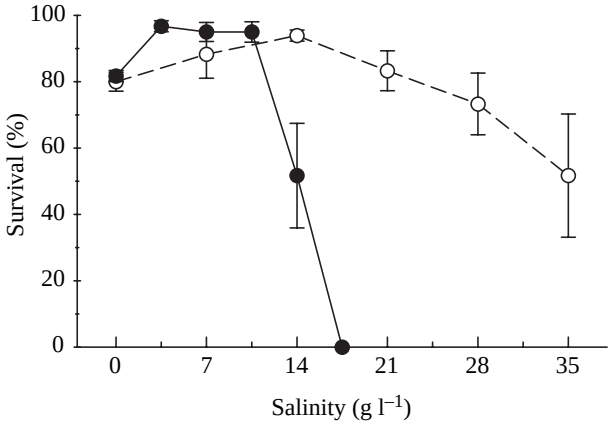

Fig. 1. Effects of salinity on successful pupation rates of larval *Aedes aegypti* (solid line) and *Ochlerotatus taeniorhynchus* (broken line). *N*=3 replicates/species, each replicate consisting of 20 larvae/salinity for a total sample size of 360 larvae of each species. Data are expressed as raw means ± S.E.M. for the percent survival of the 3 replicate runs.

Effects of species, sex and salinity on life-history variables

Aedes aegypti was reared at salinity intervals of 3.5 g l^{-1} whereas *O. taeniorhynchus* was reared at intervals of 7 g l^{-1}. Two response variables were measured: duration of the larval stage and pupal mass (wet and dry). From these parameters the mean growth rates of wet and dry mass over the larval stage were calculated. Mixed linear models run on a reduced data set including only shared salinities show that the two species differ with respect to all five life-history variables (main effect of species, $P<0.01$ for all life history response variables). In addition, the dependence of these variables on sex and salinity is significantly affected by species [sex×species, $P<0.01$ for mass and wet growth rate; salinity×species, $P<0.0001$ for ln(days to pupation); salinity squared×species, $P<0.005$ for wet and dry growth rate and log-transformed days to pupation]. Separate models were therefore used to describe each species (Table 1).

Duration of the larval stage

Within a species, duration of the larval stage is sex-dependent (Fig. 2; Table 1). Both sexes respond to salinity in a similar way, but females consistently take longer to pupate than do males. Distinct patterns of response to salinity are observed between species. The duration of the larval stage of *O. taeniorhynchus* is positively related to salinity across the entire range tested (0–35 g l^{-1}) (Table 1). Changes in duration of the larval stage due to salinity are less than 10% of the total. The pattern observed in larval *A. aegypti* is quite different. Developmental rates of these larvae show a U-shaped curve, with most rapid development occurring at 7 g l^{-1} salinity, and developmental time increasing abruptly as salinity increases above that value (Fig. 2). This is reflected in the significant effect of the quadratic term for salinity (salinity squared) on log-transformed duration of larval stage in this species (Table 1). Male developmental duration increases by 24%, while female duration increases by 15%, as salinity increases from 7 g l^{-1} to 14 g l^{-1} (Fig. 2). This is the same range over which survival decreases precipitously (Fig. 1).

Table 1. *Best fit mixed linear models describing the effects of sex, salinity and their interactions on life history parameters of* Aedes aegypti *and* Ochlerotatus taeniorhynchus

	Aedes aegypti			*Ochlerotatus taeniorhynchus*		
Effect	n.d.f.	d.d.f.	*F* value	n.d.f.	d.d.f.	*F* value
ln(Days to pupation)						
Sex	1	336	12.1***	1	269	42.4***
Salinity	1	336	31.6***	1	269	23.0***
Salinity^2	1	336	51.9***			
Wet mass						
Sex	1	337	683.2***	1	268	272.1***
Salinity	1	337	18.6***	1	268	0 NS
Salinity^2				1	268	3.9*
Growth rate, wet mass						
Sex	1	336	399.5***	1	269	65.0***
Salinity	1	336	3.3†	1	269	2.1 NS
Salinity^2	1	336	13.2***			
Dry mass						
Sex	1	335	369.1***	1	259	179.7***
Salinity	1	335	15.8***	1	259	42.3***
Growth rate, dry mass						
Sex	1	334	210.5***	1	259	61.7***
Salinity	1	334	4.9**	1	259	9.9**
Salinity^2	1	334	14.7***			

n.d.f., numerator degrees of freedom; d.d.f., denominator degrees of freedom.

***$P<0.001$; **$0.001<P<0.01$; *$0.01<P<0.05$; $^{\dagger}0.05<P<0.1$; NS, $P>0.1$.

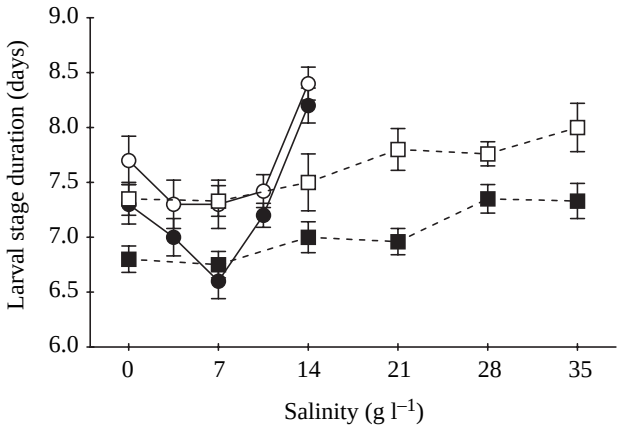

Fig. 2. Effects of salinity, species and sex on duration of the larval stage in those *Aedes aegypti* (solid line) and *Ochlerotatus taeniorhynchus* (broken line) surviving to pupation. Males, filled symbols; females, open symbols. Sample sizes of surviving larvae were as follows: *A. aegypti*: 188 males, 153 females; *O. taeniorhynchus*: 149 males, 123 females. Data are expressed as raw means ± S.EM.

Pupal mass

Clear differences are observed between species and sexes in pupal mass. Within each species females are larger than males (Fig. 3A,B; Table 1). *Ochlerotatus taeniorhynchus* is larger than *A. aegypti*. Salinity affects pupal mass in both species studied. Pupal wet and dry masses of male and female *O. taeniorhynchus* increase with salinity across the entire range of salinities tested (Fig. 3A,B; Table 1). As salinity increases from 0 to 35 g l^{-1}, in *O. taeniorhynchus* male mass increases by 18% (wet mass) and 26% (dry mass) while female mass increases by 17% (wet mass) and 25% (dry mass). The increase in wet mass in *O. taeniorhynchus* across salinities shows significant curvature (salinity^2 term, Table 1), with mass remaining relatively constant across the midrange of salinities and greater increases in mass over a given salinity increment at higher salinities. In contrast, pupal mass (wet and dry) of *A. aegypti* decreases linearly as salinity increases from 0 to 14 g l^{-1} (Fig. 3A,B; Table 1). Male wet mass decreases by 21%, while female wet mass decreases by 14%, across this salinity range. The effects of salinity on the two species thus differ both in sign and in curvature.

Growth rate

Growth rates (rates of assimilation of wet and dry mass) are strongly dependent on species and sex (Table 1, Fig. 4A,B). The effects of salinity are species dependent. Growth rates (wet and dry mass) are significantly greater in females than in males of each species (Table 1). For *O. taeniorhynchus*, dry mass growth rate increases significantly with increasing salinity, but wet mass growth rate remains constant across salinities reflecting a decrease in percent body water with salinity (P<0.001, mixed linear model including sex and salinity). In *A. aegypti*, growth rates of wet and dry mass both decrease significantly with salinity and this decrease is accelerated at the greatest salinities (Table 1; salinity^2). Salinity has no significant effect on the percent body water of *A. aegypti* (P=0.61, mixed linear model, including sex and salinity).

Differences in patterns of growth and development

The differences in the effects of salinity on growth and development in these two closely related species are summarized in the path diagrams shown in Fig. 5. The difference in sign of the effect of salinity on growth rate in the

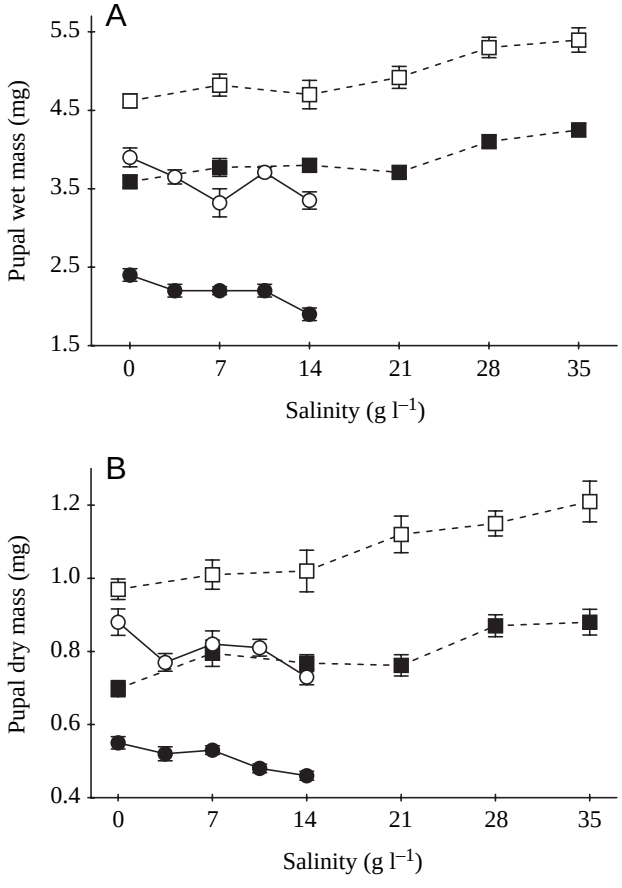

Fig. 3. Effects on salinity, species and sex on pupal mass of *Aedes aegypti* (solid line) and *Ochlerotatus taeniorhynchus* (broken line) surviving to pupation. Effects on (A) wet mass and (B) dry mass. Males, filled symbols; females, open symbols. Sample sizes are as in Fig. 2 legend. Data are expressed as raw means ± S.E.M.

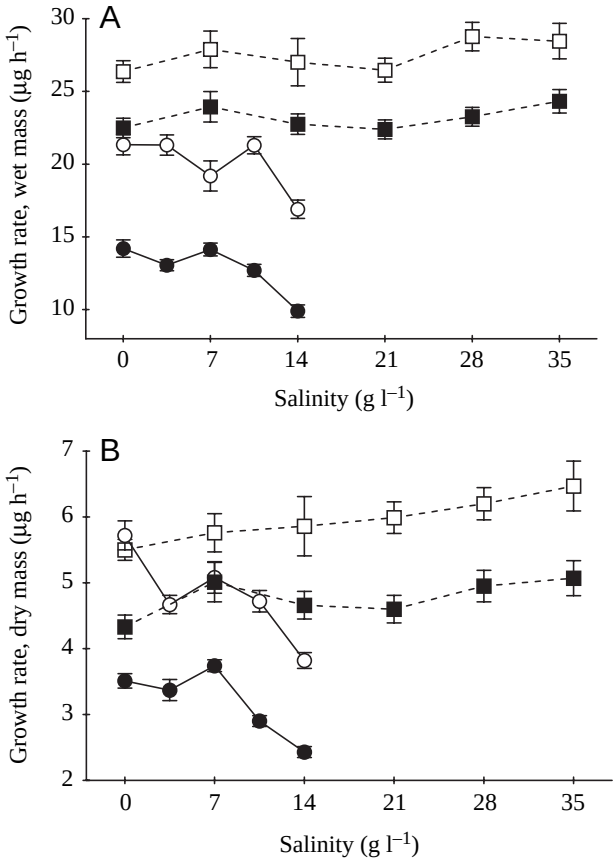

Fig. 4. Effects of salinity, species and sex on growth rate of *Aedes aegypti* (solid line) and *Ochlerotatus taeniorhynchus* (broken line) surviving to pupation. Effects on (A) growth rate (wet mass), (B) growth rate (dry mass). Males, filled symbols; females, open symbols. Sample sizes are as in Fig. 2 legend. Data are expressed as raw means ± S.E.M.

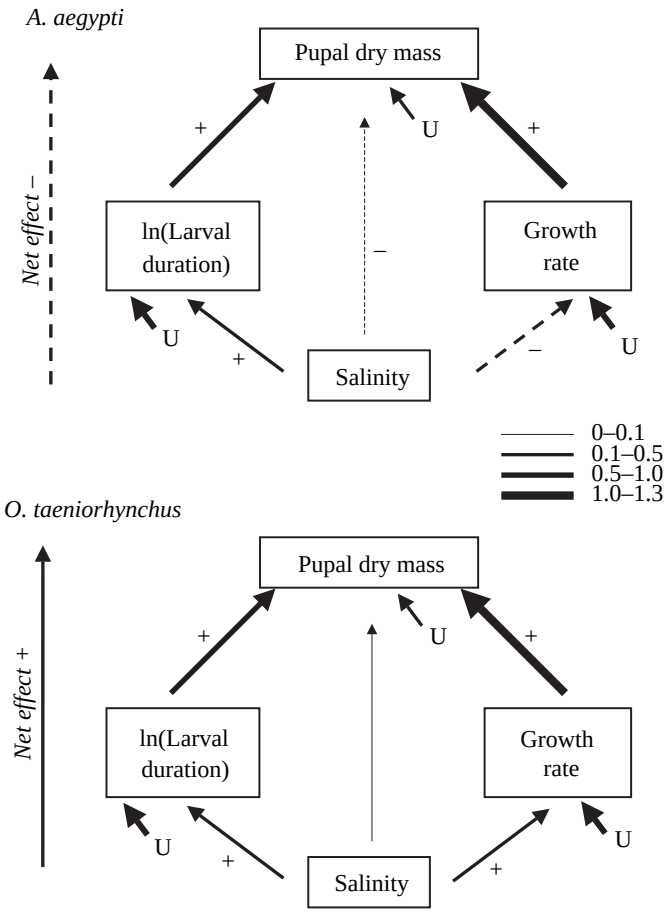

Fig. 5. Path diagrams of the relationship among salinity, growth parameters and final pupal dry mass in *Aedes aegypti* and *Ochlerotatus taeniorhynchus*. Solid lines indicate positive and broken lines negative path coefficients; the values of the path coefficients are indicated by line thickness. Coefficients that are not significant at $P<0.05$ are not shown. U, unexplained variance. The univariate (net) coefficient describing the overall effect of salinity on pupal dry mass is shown by the vertical arrows at far left of the figure.

two species explains much of the final difference in the effects of salinity on pupal dry mass, a major fitness component, since growth rate is strongly positively correlated with mass at pupation. In *O. taeniorhynchus* pupal dry mass is highest at high salinity (Fig. 3). Multiplication of the path coefficients along each side of the diagram reveals that 40% of this correlation can be explained by salinity's effects *via* the time to pupation, while most of the remaining 60% of the explained variability is due to its effects on dry growth rate. In contrast, the net effect of increasing salinity for *A. aegypti* is a decrease in pupal dry mass (Fig. 3), mainly through its effect of decreasing growth rate, as 70% of the explained variability in dry mass at pupation is *via* the negative effects of salinity on assimilation of dry mass. The increased larval duration that occurs at high salinity explains close to 30% of the variability in pupal dry mass, but does not fully compensate for the negative effect of salinity on dry growth rate.

Discussion

Holometabolous insects including mosquitoes, with distinct larval, pupal and adult stages, show clearly the difference between growth and development. Growth describes increases in size or mass, while development describes changes in which physiological and morphological characteristics progress toward reproductive maturity (Butler, 1984). These two processes are related, as pupation typically does not occur until the larvae attain a certain minimum size or, more importantly, a critical level of nutrient stores (Clements, 2000). In mosquitoes, as in many other insects, adult fecundity and reproductive success are influenced by size. Size in insects has a strong genetic component but can also be influenced to a variable degree by environmental conditions. Because adults are enclosed in an exoskeleton and do not molt, adult size and dry mass are determined in large part by the dry mass of the larvae at the time of pupation. This is in turn determined by the ability of the larvae to acquire and conserve nutrients and the length of the larval stage. Any stress that increases energy expenditure or reduces nutrient assimilation efficiency will necessarily increase feeding rates, reduce adult size and nutrient stores, lengthen the larval stage, or cause a combination of these effects.

The different physiological mechanisms used by these two species to deal with ionic loads are expected to lead to differences in the effects of salinity on the balance between nutrient assimilation and nutrient expenditure during development. This is expected to lead in turn to differences in responses of growth and developmental parameters to salinity in the two species. We originally hypothesized that the two species would react in a similar way at low salinities, and differences would emerge and increase in magnitude as the salinity departed from values normally experienced by freshwater larvae. What we found instead is that the developmental programs of the two species respond to salinity in fundamentally different ways. The effects of salinity on growth and development of *O. taeniorhynchus* are due to positive direct influences of salinity on assimilation of dry mass, and on larval stage duration, both of which positively influence mass at pupation (Fig. 5). Although the increase in larval stage duration in response to increased salinity is actually larger than the increase in dry growth rate (Figs 2, 4), because the latter so strongly influences pupal mass, it is *via* its effect on growth rate that salinity most strongly influences final dry mass (Fig. 5). In *O. taeniorhynchus*, pupal mass is a function of larval stage duration, which is positively related to salinity (see Figs 3, 5). Thus, it appears that in *O. taeniorhynchus* the decision to pupate is uncoupled from information about pupal mass, so that increased larval stage duration and growth rate results in greater mass as salinity increases. In *A. aegypti*, a very different pattern emerges. Salinity influences mass through a curvilinear effect on larval duration, which lengthens the time to pupation at high salinities (Table 1, Figs 2, 5). However increased salinity also decreases larval growth rate in *A. aegypti* (Table 1, Figs 4, 5). As a result, despite the positive correlation between increased larval duration and mass at pupation, the increase in this trait at

high salinity cannot fully compensate for negative effects of salinity *via* growth rate, resulting in a net negative effect of salinity on pupal dry mass (Table 1, Figs 2, 5). As salinity increases above 7 g l^{-1}, *A. aegypti* may partially compensate for salinity-induced changes in growth rates by adjusting developmental time, delaying pupation until the animal has acquired the critical mass for pupation and thereby maintaining pupal mass. The overall negative slope of this relationship shows that larvae in more saline water delay pupation but still pupate before they attain the mass that they would have attained in less saline conditions. This pattern suggests that *A. aegypti* assesses both larval stage duration and mass, reaching a compromise between rapid completion of the larval stage and maintaining ideal mass. Salinity thus influences developmental programs in fundamentally different ways in the two species investigated.

The increment in salinity that most dramatically delays development of *A. aegypti* is that over which the hemolymph osmotic pressure approaches the osmotic pressure of the environment. In larval mosquitoes, feeding leads to ingestion of the medium and may thus contribute to ionic loads under saline conditions. The decrease in growth rate of *A. aegypti* at greater salinities (Table 1, Fig. 4) may be due to decreased feeding rates to avoid ingestion of ions at greater rates than can be eliminated by the excretory system, and/or by decreased assimilation of nutrient stores due to increased metabolic demands of osmo- and iono-regulation at elevated salinities. This pattern could be explained by relatively slow growth rates at the time that they reach the critical mass for pupation, so that pupation occurs before significant additional mass has been accumulated. These data also suggest that a significant portion of the energy budget of larval *A. aegypti* is used for ionoregulation at higher salinities within the tolerable range.

In situations where the commitment to pupate occurs at a given mass, larger size together with delayed development, as occurs in *O. taeniorhynchus* (Table 1, Fig. 2), should occur if environmental conditions delay early growth until physiological adjustments have been made. If growth is especially rapid following these adjustments, then given fixed times between reaching critical mass and pupation, the mass of the insect would overshoot the target. Intriguingly, these data resemble those describing the relationship between adult size and developmental temperatures, in which larvae develop more slowly at lower temperatures but reach greater size (Clements, 2000).

The trade-off that exists between completing development quickly and attaining large size appears to lead to selection for increased growth rates in *O. taeniorhynchus* relative to *A. aegypti*, and in females of both species relative to males. It is tempting to speculate that the differences between the growth rates of *A. aegypti* and *O. taeniorhynchus* (Figs 2–4) are evolved traits driven by selective pressures. This is supported by the observation that the differences in growth rates of the two species are paralleled by differences in the behavior of their larvae and pupae. Larvae and pupae of *O. taeniorhynchus* swim quite rapidly, darting around the container in rapid bursts. *A. aegypti* larvae on the other hand swim slowly, and have a much greater tendency to mass together in the darkest corner of the rearing dish (T. M. Clark, unpublished observation). *Ochlerotatus taeniorhynchus* also completes the pupal stage within 48 h at 26°C, whereas the pupal stage of *A. aegypti* typically lasts more than 48 h at this temperature (T. M. Clark, unpublished observation). The reason for greater pupal mass of *O. taeniorhynchus* is likely to involve selective pressures acting on adults, such as selection for increased fecundity, possibly driven by increased mortality of larvae in their less-protected larval habitats. Similarly, the differences in growth rates and swimming speeds are likely to be driven by different selective pressures experienced by larvae of the two species in their natural habitats. *Ochlerotatus taeniorhynchus* larvae live in salt marshes, where predators such as fishes are common and rapid evaporation of temporary pools occurs. Larval *A. aegypti*, on the other hand, live in very small bodies of water such as tin cans and discarded tires, habitats unlikely to contain such predators. The difference in growth rates between the species increases with increasing salinity reflecting their different mechanisms of iono- and osmo-regulation.

A large number of environmental parameters influence growth and development in larval mosquitoes. These sources of variability may explain some of the discrepancies between the results of the current study and those of Nayar (1969), who observed that size, dry mass and percent lipid of *O. taeniorhynchus* decrease with increasing salinity. It is possible, but unlikely, that the differences are due to evolution of the laboratory strain used in the two studies during the 30+ years since the studies of Nayar (1969). The differences between the results of that study and the present one more probably result from different rearing conditions, such as feeding regimens. Similarly, the significance of comparisons between the present work and the work of McGinnis and Brust (1983) is not clear. In their study, the euryhaline *Aedes togoi* showed a U-shaped response of developmental time to salinity, similar to the response of *A. aegypti*. However, unlike both *A. aegypti* and *O. taeniorhynchus* (this study), *A. togoi* showed the slowest development in the most dilute water. It is possible that this species exhibits yet a third pattern of response although once again we suspect that environmental conditions contribute to the observed differences.

This is the first study to directly compare growth and development of two species under the same environmental conditions in order to avoid such artifacts. Patrick et al. (2002a,b) have documented surprisingly diverse mechanisms of ionoregulation among freshwater species, and even among populations within a species. The present study shows that fundamental differences in mechanisms by which growth and development respond to environmental influences can also occur among closely related species.

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