Larval environmental stress alters Aedes aegypti competence for Sindbis virus

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Summary

OBJECTIVE  To evaluate how stress at the larval stage alters adult mosquito performance and susceptibility to viral infection.

METHODS  We used a model system consisting of Sindbis virus (SINV) and the yellow fever mosquito Aedes aegypti. Larvae were either reared under optimal conditions (control) or exposed to one of four types of stressors; suboptimal nutrients, starvation, elevated temperature, and a low dose of the insecticide malathion and adult females were fed SINV infectious blood meal. Differential expressions of stress, immune-specific and detoxification genes was measured in fourth instar larvae (HSP70, HSP83, cecropin, defensin, transferrin and CYP6Z6) and 3-day-old females (cecropin, defensin, transferrin) to identify plausible molecular mechanisms associated with mosquito response to stress.

RESULTS  There were stress-specific variations in mosquito performance (survival, development time, female size), but all stressors had a consistent effect of significantly increasing susceptibility to viral infection and dissemination relative to the controls. Three genes were up-regulated in fourth instar larvae exposed to temperature stress (cecropin, defensin and CYP6Z6) compared to single genes in suboptimal nutrient (cecropin) and malathion (transferrin) stress treatments and down-regulation of all the six genes in starvation treatments. In adult samples, transferrin was up-regulated in all but starvation treatments while defensin was up-regulated in starvation and temperature stress treatments.

CONCLUSIONS  Stress during larval development may cause alterations in adult mosquito phenotype and immunity that can increase their susceptibility to pathogens.

keywords  environmental stress, vector competence, arbovirus, Sindbis, Aedes aegypti

Introduction

Understanding environmental determinants of disease transmission is one of the most pressing challenges faced by research and public health scientists, especially with the recent emergence and re-emergence of infectious diseases. In some instances, these environmental determinants are anthropogenic as is the case in tropical deforestation, irrigated agriculture, urbanization, and global climate change (Hunter 1992; Martens et al. 1995; Vora 2008). For vector-borne diseases, environmental factors may weigh heavily on arthropod vectors, in part because they determine vector abundance, fitness and competence for pathogens (Gimnig et al. 2002; Alto et al. 2005; Muturi et al. 2010) as well as the duration of the extrinsic incubation period (EIP), the time between acquisition of an infectious blood meal and when transmission is possible (Reeves et al. 1994). Currently, vector control is one of the main pillars of reducing the burden of vector-borne diseases and its success relies on proper understanding of ecological interactions between pathogens and vectors.

Some vectors such as mosquitoes have complex life cycles where oviposition and subsequent growth and development of immature stages occur in aquatic environments and the resulting adults disperse to an entirely different ecological niche in the terrestrial environment. Most investigations of interactions between parasites and vectors have focused on the environmental conditions experienced by the adult stage of the vector. However, it has become clear that the conditions experienced by immature stages of vectors may be equally, or in some instances, more important in determining the transmission of vector-borne diseases. For instance, ecological processes acting on the larval stages can significantly influence population dynamics (e.g. density-dependent regulation) and community structure (Juliano 2007; Alto et al. 2009)
of mosquito vectors as well as their interactions with pathogens (Alto et al. 2005; Bevins 2008).

Variation in temperature, intra- and interspecific competition, predation, drying of the larval habitat and chemical contaminants are some of the biotic and abiotic factors encountered by aquatic stages of mosquitoes. These factors have significant impacts on larval survivorship and development as well as on the size of subsequent adults (Bayoh & Lindsay 2003; Muturi et al. 2010). Larval rearing temperature, competition and chemical contaminants have also been associated with alterations in adult longevity (Reiskind & Lounibos 2009; Muturi et al. 2010) and susceptibility to pathogens (Alto et al. 2005; Westbrook et al. 2010; Muturi et al. 2011), both important parameters in determining transmission. However, studies investigating the impact of larval stressors on vector competence focus on single stressors at a time, making it difficult to predict their relative impact on mosquito phenotype, including vector competence for pathogens. The latter is a measurement of susceptibility to infection and transmission of pathogens. Furthermore, previous studies often hypothesize, but rarely test, the mechanisms by which environmental factors experienced by immature stages influence adult mosquito fitness and vector competence.

Differential gene expression is one of the principal mechanisms for a genotype to modify a phenotype in order to maintain high performance across a range of environments (Pigliucci 1996). Environmental stressors such as infection, exposure to toxins and pollutants, starvation, dehydration, oxygen deficiency and extreme temperatures are known to change expression of various heat shock proteins, HSPs (Tabachnick & Powell 1979; Benoit et al. 2009a,b; Pridgeon et al. 2009). Under stressful conditions, these proteins stabilize denaturing proteins and refold proteins that have already been denatured whereas under normal conditions they help proteins fold correctly during translation and facilitate their transport across membranes (Hightower 1991). The high degree of sensitivity of HSPs to multiple environmental factors has led to their utility as biomarkers of stress (de Pomeraí 1996). Production of heat shock proteins is an energy-consuming process and can deplete the cell of the energy available for critical metabolic processes such as growth and immunity (Kultz 2003). Little is known about the latent effects of heat shock response on organisms but a handful of studies suggest that it may impair fitness (Feder et al. 1996; Krebs & Feder 1997; Silbermann & Tatar 2000).

Larval environment can also alter insect immunity against pathogens as well as transcription of detoxification enzymes but this has only been demonstrated for a limited number of stressors. When larvae of Anopheles gambiae were reared in water with unautoclaved clay, they were more susceptible as adults to malaria parasites compared to adults from larvae that were reared in water with sterilized clay (Okech et al. 2007). Intra- and interspecific larval competition increased susceptibility of Aedes albopictus and Aedes aegypti to dengue and Sindbis viruses (Alto et al. 2005, 2008; Muturi et al. 2011) and Aedes triseriatus to Lacrosse virus (Bevins 2008). In some insect-pathogen systems, increases in temperature reduce susceptibility to infection (Elliot et al. 2002; Westbrook et al. 2010) while, in others, high temperature (Muller & Schmid-Hempel 1993) and heat shock treatments (Mourya et al. 2004) increase susceptibility to infection. In Drosophila melanogaster, larval food quality affects adult but not larval immune gene expression (Fellous & Lazzaro 2010). Differential expression of transcripts for detoxification enzymes in response to a variety of natural and synthetic xenobiotics (Poupardin et al. 2008, 2010) and infection with malaria parasites (Felix et al. 2010) has also been demonstrated in mosquitoes.

The current study evaluated the impact of four environmental stressors: suboptimal nutrients, starvation, exposure to sublethal concentrations of insecticides and elevated temperature on adult mosquito performance and susceptibility to virus infection. Our aim was to test three related hypotheses that larval environmental conditions induce stress-specific responses in mosquitoes manifested as differences in (i) performance, (ii) susceptibility to infection and transmission, and (iii) expression of stress- and immune-specific genes. We tested these hypotheses using a model system consisting of the yellow fever mosquito A. aegypti L. and Sindbis virus (SINV, MRE16 strain). SINV is an Alphavirus (family Togaviridae) related to more pathogenic viruses including Chikungunya, western equine encephalitis and eastern equine encephalitis viruses.

Materials and methods

Life history and vector competence studies

The experiments were conducted using the F4 generation of A. aegypti from field collections in Florida reared at 12:12 light to dark photoperiod and 70% relative humidity. 200 first instar larvae (approximately 12 h old) were added to 1.6 l of oak-leaf (Quercus virginiana) infusion held in 5 l plastic containers. The infusion was prepared by fermenting a mixture of 500 g of oak leaves and 100 l of tap water for 2 weeks. The larvae were then exposed to four types of environmental stressors, including elevated temperature, a low dose of the insecticide malathion, suboptimal nutrients and starvation, and compared with control larvae.
reared at the same time. Suboptimal nutrient and starvation treatments differed in the timing at which mosquito larvae were supplemented with minimal nutrients. At the beginning of the experiment, the containers were supplemented with larval food (1:1 yeast:albumin) as follows: 0.2 g for control, malathion and temperature treatments, 0.1 g for suboptimal nutrient treatments and none for starvation treatments. For temperature treatments, larvae were maintained at 32 °C while, for all other treatments, larvae were maintained at 25 °C. On day 8, treatments were supplemented with 0.2 g (control, malathion and temperature) or 0.1 g (suboptimal nutrient and starvation) of yeast: albumin. The malathion treatment received 4 ml of 20 mg/l malathion (final concentration of 0.05 mg/l) while the other treatments received 4 ml deionised water at the beginning of the experiment. Malathion was chosen for this study because it is widely used to control agricultural and public health pests including mosquitoes and is also a common source of water pollution with environmental concentrations of up to 1 mg/l (U.S. Department of Agriculture 1997; Relyea 2004). There were five replicates for control, starvation and suboptimal nutrient treatments and six replicates for elevated temperature treatments differed in the timing at which mosquito larvae were supplemented with minimal nutrients. At the beginning of the experiment, the containers were supplemented with larval food (1:1 yeast:albumin) as follows: 0.2 g for control, malathion and temperature treatments, 0.1 g for suboptimal nutrient treatments and none for starvation treatments. For temperature treatments, larvae were maintained at 32 °C while, for all other treatments, larvae were maintained at 25 °C. On day 8, treatments were supplemented with 0.2 g (control, malathion and temperature) or 0.1 g (suboptimal nutrient and starvation) of yeast: albumin. The malathion treatment received 4 ml of 20 mg/l malathion (final concentration of 0.05 mg/l) while the other treatments received 4 ml deionised water at the beginning of the experiment. Malathion was chosen for this study because it is widely used to control agricultural and public health pests including mosquitoes and is also a common source of water pollution with environmental concentrations of up to 1 mg/l (U.S. Department of Agriculture 1997; Relyea 2004). There were five replicates for control, starvation and suboptimal nutrient treatments and six replicates for elevated temperature and malathion treatments.

Each of the containers was examined daily and pupae were placed in plastic vials with cotton seals. Each day, emerging adults were transferred by replicate into new paperboard cages (11 cm high · 9.5 cm diameter) and continuously provided with 0.2 g (control, malathion and temperature) or 0.1 g (suboptimal nutrient and starvation) of yeast: albumin. The malathion treatment received 4 ml of 20 mg/l malathion (final concentration of 0.05 mg/l) while the other treatments received 4 ml deionised water at the beginning of the experiment. Malathion was chosen for this study because it is widely used to control agricultural and public health pests including mosquitoes and is also a common source of water pollution with environmental concentrations of up to 1 mg/l (U.S. Department of Agriculture 1997; Relyea 2004). There were five replicates for control, starvation and suboptimal nutrient treatments and six replicates for elevated temperature and malathion treatments.

Each of the containers was examined daily and pupae were placed in plastic vials with cotton seals. Each day, emerging adults were transferred by replicate into new paperboard cages (11 cm high × 9.5 cm diameter) and continuously provided with 10% sucrose, allowing us to keep track of their age, survival rates and development times to adulthood. Six- to 10-day-old females were sugar-starved for 48 h and then allowed to blood-feed on an artificial membrane feeding system (Hemotek, Lancashire, UK) for 30 min on citrated bovine blood containing 10³ plaque-forming units/ml of SINV (MRE16 strain from Sindbis Health District, Nile Delta, Egypt). Each plaque-forming unit is assumed to have originated from infection by a single virus. Unfed females were provided a second chance to blood feed on SINV-infected blood the following day and females that did not blood-feed were killed and their wings measured (using iSolution lite, IMT i-solution, Inc., Vancouver, Canada) as an indicator of size. Blood-fed females were transferred into new cages, maintained at 25 °C and provided continuous access to an oviposition cup and 10% sucrose for a 14-day virus incubation period. After the incubation period, females were killed and stored individually stored at -80 °C. The wings of these mosquitoes were measured as an indicator of size.

The bodies and legs were homogenized in 1.0 ml of cell culture media (Leibovitz L-15 media, 10% foetal bovine serum (FBS), 1% penicillin/streptomycin) and 220 µl of mosquito body or legs homogenate were used for RNA extraction using Qiamp® virus Biorobot® 9604 kit according to manufacturer’s protocol (Qiagen). The resulting RNA was redissolved in 80 µl TE buffer and stored at -80 °C prior to use. RNA from body and leg samples were assayed for SINV using Taqman probe RT-PCR to determine the infection status and disseminated infection, respectively. PCR amplification was conducted in 25 µl reactions containing 12.5 µl 2x one-step RT-PCR master mix (Applied Biosystems), 0.65 µl RNase inhibitor (Qiagen), 0.25 µl of each 10 µM forward and reverse primer stock, 0.125 µl 10 µM Taqman probe, 6.2 µl nuclease-free water (Integrated DNA Technologies, Inc.) and 5 µl template RNA. Primers and probe used for this study were: forward primer (5'-CACWCCAAATGAC CATGC-3'), reverse primer (5'-KGTGCTCGGAAWAC ATTC-3'), and probe (5'-FAM-CAGACGTATT CGCATCTGGC-BHQ1-3'). RT-PCR reactions were conducted in a 7300 real time PCR system (Applied Biosystems) using the following thermocycles: 50 °C for 30 min, 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min.

Using SAS statistical package (SAS Institute 2002) data were checked for normality and homogeneity of variances and analysed using one-way analysis of variance (ANOVA) for survivorship (females + males), size and development time of females. When a significant treatment effect was detected, pairs of treatment means were compared using the Ryan–Einot–Gabriel–Welsch multiple range test. Multivariate analysis of variance (MANOVA) was used to test for treatment effects on SINV infection and dissemination rates. Standardized canonical coefficients were used to determine the relative contribution and relationship between infection parameters (infection, dissemination) to a significant treatment effect (Scheiner 2001).

Gene expression studies

An experiment identical to the one previously described was conducted to determine the impact of larval treatments on expression of HSPs (HSP70 and HSP83), CYP6Z6, antimicrobial peptides (defensin and cecropin) and trans-ferrin. However, mosquitoes were not provided with an infectious blood meal. Rather, RNA for gene expression was extracted from fourth instar larvae and 3-day-old adult females using Qiamp® virus Biorobot® 9604 kit according to manufacturer’s protocol (Qiagen). RNAs were quantified using nanodrop readings and 500 ng total RNA were treated with U DNase 1 (Biolabs) in 20 µl reactions containing 10 µl 1x one-step RT-PCR master mix, 0.5 µl 40× multiscrube and
RNase inhibitor mix, 0.5 μl of each 10 μM forward and reverse primer stock, 1 μl 20x SYBR Green dye, 5.5 μl double distilled water and 2 μl template RNA. RT-PCR thermocycling conditions were 50 °C for 60 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s (defensin, cecropin, transferrin and HSP70) and 50 °C for 60 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 50 °C for 30 s, and 72 °C for 30 s (HSP83 and CYP6Z6). Gene-specific primers used in this study are listed in Table 1. Relative gene expression was performed according to 2^ΔΔCT method (Livak & Schmittgen 2001) and normalized using ribosomal protein L8 (AeRPL8) and actin respectively for larval and adult data. For all treatments, there were four biological and three experimental replicates. Results are expressed as mean expression ratios (±SE) between larvae exposed to each stress and controls with genes showing at least twofold over- or underexpression in larvae or adults considered to be differentially expressed.

Results

Effect of larval stress on life history traits

Treatments significantly affected survivorship (males + females), female size, and female development. A. aegypti survival was highest in control and temperature treatments, intermediate in suboptimal nutrients treatment and lowest in malathion and starvation treatments (F_{4,18} = 118.29, P < 0.0001; Figure 1a). For the size effect, mosquitoes surviving exposure to malathion had significantly longer wings compared to all other treatments, including controls (F_{4,18} = 86.28, P < 0.0001; Figure 1b). Exposure to suboptimal nutrients, starvation and elevated temperature resulted in significantly shorter wings relative to controls (Figure 1b). Female development times were shortest in malathion and temperature treatments, intermediate in the controls and longest in starvation and suboptimal nutrients treatments (F_{4,18} = 94.02, P < 0.0001; Figure 2).

Effect of larval stress on vector competence

MANOVA showed a significant treatment effect on susceptibility to infection and dissemination of SINV (Pillai’s trace_{8,44} = 1.11, P < 0.0001). Standardized canonical coefficients were positive and differences in mosquito infection contributed approximately 35% more of the variation relative to dissemination (infection SCC, 1.79; dissemination SCC, 0.62). Thus, both infection and dissemination responded in a similar direction to the treatment effect but had somewhat different contributions. In all instances, susceptibility to virus infection and dissemination were significantly lower in controls relative to all treatment stressors (Figure 3). Exposure to suboptimal nutrients, starvation, and malathion in immature stages resulted in adults with the highest infection, with exposure to elevated temperature resulting in adults with

Table 1 Primers used for quantitative real-time PCR experiments

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>CYP6Z6</td>
<td>Forward: 5-CTGCCCTATTGGACCTATATGC-3</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Forward: 5-CCCGTTCTACGTGGCGATCTA-3</td>
</tr>
<tr>
<td>Hsp26</td>
<td>Forward: 5-TTGACATCTCTTCTCCGTT-3</td>
</tr>
<tr>
<td>Hsp83</td>
<td>Forward: 5-AAGGCCGTATGGATCTGTT-3</td>
</tr>
<tr>
<td>Defensin</td>
<td>Forward: 5-GCACTCTGTCATCTGCTGGCAGA-3</td>
</tr>
<tr>
<td>cecropin A</td>
<td>Forward: 5-ATTCCCTATGCTTGCCCTGGT-3</td>
</tr>
<tr>
<td>transferrin</td>
<td>Forward: 5-CGTCGGCTATGGATCTGTCGCTG-3</td>
</tr>
<tr>
<td>AeRPL8</td>
<td>Forward: 5-TGGGGCGTGTTATTCGTGCAAG-3</td>
</tr>
</tbody>
</table>

Figure 1 LS means (±SE) (a) survivorship (males + females) and (b) female wing length. Means followed by different lower case letters show significant differences for pair-wise comparisons.
somewhat lower susceptibility to infection (Figure 3). There were no significant differences in SINV dissemination among treatment stressors (Figure 3).

Effect of larval stress on larval and adult gene expression

The relative expression pattern of six genes in fourth instar larvae in response to larval stress is shown in Table 2. All of the genes were under-expressed in starvation treatments relative to control treatments. HSP83 was also 3.0-fold under-expressed in malathion and temperature stress treatments. Expression of cecropin was 2.2- and 2.8-fold higher, respectively, in suboptimal nutrients and temperature stress treatments relative to control treatments. Transferrin and CYP6Z6 were 2.0- and 2.4-fold overexpressed, respectively, in malathion and temperature treatments.

In adults, there was at least 2-fold overexpression of transferrin in suboptimal nutrients, malathion and temperature stress treatments relative to control treatments and of defensin in starvation and temperature treatments. Cecropin was not overexpressed in any of these treatments (Figure 4).

Discussion

Our study shows that different environmental stressors experienced by A. aegypti larvae have variable effects on adult life history traits and vector competence. Starvation and presence of malathion were the most lethal stressors followed by suboptimal nutrients treatment while temperature stress had no discernible effects on survival. We also observed differences in development time in response to the four environmental stressors that were examined. As previously reported, malathion and temperature stress resulted in shorter development time to adulthood while suboptimal nutrients and starvation treatments resulted in longer development times (Bayoh & Lindsay 2003; Muturi et al. 2010). Shorter development time in the presence of chemical contaminants and high temperature is beneficial to surviving mosquitoes as it may reduce the duration of larval exposure to chemicals and the risk of desiccation, respectively. Conversely, in the absence of other mortality factors (e.g. predation, desiccation), longer development

Figure 2 LS means (±SE) for female development time to adulthood. Means followed by different lower case letters show significant differences for pair-wise comparisons.

Figure 3 LS means (±SE) percent infection and dissemination of Sindbis virus grouped by treatment. Numbers associated with means represent the total mosquitoes tested. Means followed by different lower and upper case letters show significant differences for pair-wise comparisons.
time under suboptimal food conditions may increase the chances of survival because mortality of individuals that are less resistant to starvation may release some pressure on shared resources (Barrera 1996). In addition, mosquito larvae that persist under resource-limited conditions may benefit from subsequent inputs of food resources in these habitats (e.g. leaf fall; Barrera 1996).

Larval environmental stressors also caused alterations in size of adult females. Exposure to malathion resulted in larger adults relative to other treatments while the other stressors resulted in smaller adults, with suboptimal nutrient treatments producing the smallest adults. Because adult body size is positively related to other life history traits such as longevity (Reiskind & Lounibos 2009) and fecundity (Briegel 1990), our finding suggest that constraints on mosquito fitness may be specific to the type of environmental stress. Malathion-mediated killing of some larvae may have released the survivors from competition, resulting in shorter development time and larger adults (Antonio et al. 2008). It is also possible that malathion selected for larger mosquitoes with faster development but our experimental design could not distinguish between the two mechanisms (plasticity vs. selection).

Partial support for release from competition was also observed in the starvation treatment, in which lower survivorship and larger females were obtained compared to the suboptimal nutrient treatment. Shorter development time to adulthood and small adult body size in the temperature stress treatment are consistent with findings of previous studies (Bayoh & Lindsay 2003, 2004). Higher temperatures support rapid development of mosquito larvae and this may reduce their effectiveness to acquire and accumulate nutrient reserves (Korochkina et al. 1997).

Table 2  Relative gene expression in response to different types of stress

<table>
<thead>
<tr>
<th>Stress</th>
<th>Cecropin</th>
<th>Defensin</th>
<th>HSP70</th>
<th>HSP83</th>
<th>Transferrin</th>
<th>CYP6Z6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suboptimal*</td>
<td>2.17 ± 0.18</td>
<td>1.55 ± 0.25</td>
<td>1.09 ± 0.20</td>
<td>1.56 ± 0.54</td>
<td>0.94 ± 0.23</td>
<td>0.98 ± 0.33</td>
</tr>
<tr>
<td>Control</td>
<td>1.01 ± 0.10</td>
<td>1.03 ± 0.14</td>
<td>1.02 ± 0.10</td>
<td>1.13 ± 0.29</td>
<td>1.04 ± 0.16</td>
<td>1.35 ± 0.60</td>
</tr>
<tr>
<td>Malathion</td>
<td>1.44 ± 0.51</td>
<td>1.49 ± 0.36</td>
<td>0.85 ± 0.09</td>
<td>0.33 ± 0.10</td>
<td>2.09 ± 0.80</td>
<td>1.90 ± 0.90</td>
</tr>
<tr>
<td>Starvation</td>
<td>0.15 ± 0.08</td>
<td>0.56 ± 0.17</td>
<td>0.03 ± 0.00</td>
<td>0.06 ± 0.03</td>
<td>0.42 ± 0.28</td>
<td>–</td>
</tr>
<tr>
<td>Temperature</td>
<td>2.78 ± 1.03</td>
<td>2.69 ± 1.19</td>
<td>1.50 ± 0.20</td>
<td>0.30 ± 0.03</td>
<td>1.84 ± 0.62</td>
<td>2.39 ± 0.90</td>
</tr>
</tbody>
</table>

Gene expression was measured by real-time quantitative RT-PCR (qRT-PCR) in fourth instar larvae from different stress treatments. Gene expression values are indicated as fold expression (±SE) in fourth instar larvae exposed to each stress compared to unexposed larvae (controls). The housekeeping gene AeRPL8 was used as internal control for normalization.

*Suboptimal nutrients.
Dash (–) indicate that the PCR product was not detected.

Figure 4  Relative gene expression in A. aegypti adult females in response to different types of stress. The housekeeping gene AeActin was used as internal control for normalization. Horizontal dotted line indicates a twofold up-regulation in treatment groups relative to controls.
Vector competence studies indicated that irrespective of type, stress increased SINV infection and dissemination rates. Suboptimal nutrients, starvation and malathion treatments had greater impacts than temperature stress on SINV infection rate but SINV dissemination rates were similar across the four stressors. These findings suggest that environmental stress may increase the risk of arbovirus transmission. To date, there is no consensus on how environmental stressors evaluated in the current study affect vector competence. Some studies have shown that these stressors may reduce vector competence (Yadav et al. 2005; Westbrook et al. 2010) while others suggest that they could increase vector competence (Grimstad & Walker 1991; Alto et al. 2005; Richards et al. 2007; Muturi et al. 2011). The mechanism(s) in which these stressors act to increase vector competence are poorly understood. The breakdown of the midgut escape barrier has been suggested as the mechanism underlying increased vector competence in small mosquitoes from highly competitive larval environment (Grimstad & Walker 1991). Rapid development at high temperature may lead to poor development of tissues (Korochkina et al. 1997) including those involved in defence against arboviruses. Moreover, induction of detoxification enzymes by insecticides may have pleotropic effects on at least one of the steps of immune cascade including recognition of the pathogen as non-self, signal transduction and deployment of the killing mechanism (Dimoupolos 2003). These factors may increase susceptibility to arboviruses.

Organisms respond to environmental fluctuations by altering the patterns of gene expression (Snell-Rood et al. 2010). HSPs are induced by a wide range of environmental stressors and aid in stabilizing denaturing proteins and refolding those that are already denatured (Hightower 1991). In the current study, starvation treatment resulted in down-regulation of all six genes tested in the fourth instar larvae while the remaining stressors resulted in stress-specific differential up-regulation of these genes. These findings suggest that mosquito larvae may reduce their investment in defence and immunity when faced with starvation. Such a strategy may allow for allocation of the limited energy available to survival, growth and development but also may increase the probability of infection. Three genes were up-regulated in temperature stress treatments (cecropin, defensin and CYP6Z6) compared to single genes in suboptimal nutrient (cecropin) and malathion (transferrin) stress treatments. Defensins and cecropins are two of the three families of antimicrobial peptides (AMPs) identified in mosquitoes (Waterhouse et al. 2007). The former are 4 kDa polypeptides with activity against gram-positive bacteria but are also responsive to mosquito infection with different types of pathogens including bacteria, fungus, and parasites (Lowenberger et al. 1995; Magalhaes et al. 2008). Two of three defensin isoforms (A and B) reported in A. aegypti are expressed mainly in the fat body while the third isoform (C) is expressed in the midgut at a much lower level (Lowenberger 2001). Cecropins are 4 kDa polypeptides with lytic activity against both gram-positive and gram-negative bacteria. Two isoforms of cecropin (A and B) have been identified in A. aegypti and are responsive to bacteria inoculation (Lowenberger et al. 1999). Transferrin is an iron-binding protein involved in iron transport (Nichol et al. 2002) and also serves as an antimicrobial agent in insects (Yoshiga et al. 1997). CYP6Z6 is a CYP450 protein induced by numerous chemical contaminants and likely to be involved in detoxification of xenobiota. In A. aegypti, this enzyme is induced by exposure to both natural and synthetic chemicals and is believed to play a significant role in insecticide resistance development (Marcombe et al. 2009; Riaz et al. 2009). Expression of these genes is metabolically costly because it consumes the energy that would otherwise be allocated for growth and development (Silbermann & Tatar 2000; Berticat et al. 2008). This may partly account for significantly smaller adults observed in suboptimal nutrient and temperature treatments relative to the controls. Our preliminary data indicated that the lethal effects of malathion on A. aegypti were short-lived, lasting only approximately 2 days (unpublished data). Thus, while the other stressors lasted throughout the duration of aquatic stages, malathion may have acted within 2 days of application, killing a fraction of the larvae and releasing the survivors from competition. Thus, surviving larvae from malathion treatments had enough food to allow them to allocate energy for gene (transferrin) up-regulation without compromising growth and development.

Differential expression of defensin and transferrin in adult mosquitoes indicates that conditions experienced by mosquito larvae have latent effects on immunity of subsequent adults. Previous studies have shown that adult mosquitoes undergo gut-sterilization where bacteria flora associated with the larvae is ‘cleaned’ during metamorphosis and the adults acquire a new set of microbes (Moll et al. 2001). Because defensin and transferrin production is responsive to microorganisms (e.g. bacteria, fungi, viruses and parasites), our findings suggest that environmental stress may predispose adult mosquitoes to microbial infections. Whether the microbial composition between normal and stressed mosquitoes is identical remains to be examined. Also, it remains uncertain whether the observed pattern of gene expression had a role in the observed stress-mediated increase in vector competence. However, there are two lines of evidence suggestive of a close relationship between the two observations. First, prior immune
challenge of A. aegypti with a gram-negative bacteria (Escherichia coli) known to activate production of AMPs, resulted higher titres of dengue 2 (DENV-2) virus suggesting that immune deficiency (IMD) pathway (which is involved in production of AMPs) play an important role in anti-virus response (Sim & Dimopoulos 2010). The authors hypothesized that this could be due to exhaustion of cell’s capacity to produce IMD pathway-regulated effectors thus allowing rapid replication of the virus. Second, mosquitoes harbour large numbers of endosymbiotic bacteria in the midgut (Rani et al. 2009) that may serve as midgut barriers to infection (Mourya et al. 2002b). However, as in other host–pathogen systems (Xiang et al. 1999), their composition and complex interaction with mosquito hosts may be disrupted by environmental stress resulting in a compromised midgut barrier with higher susceptibility to arboviruses and opportunistic bacteria. Elimination of endosymbiotic bacteria through antibiotic treatment increased susceptibility of Anopheles mosquitoes to Plasmodium falciparum (Hoffmann et al. 1999). Similarly, addition of bacteria isolates Aeromonas calicicola and E. coli in dengue-infected blood meals increased susceptibility of A. aegypti to dengue virus (Mourya et al. 2002b). However, incorporation of Culex quinquefasciatus midgut isolates of Pseudomonas sp., Acinetobacter junii, Staphylococcus epidermis and A. calicicola in blood meals did not alter susceptibility of this mosquito species to Japanese encephalitis virus (Mourya et al. 2002a).

In summary, we observed stress-specific differential expression of antimicrobial peptides and CYP6Z6. Using a SINV model and colonized A. aegypti, we show that the effects of larval environment may continue to adulthood and alter adult life history traits and competence for arboviruses. Regardless of the type of stress, our findings indicate that environmental stress increases vector competence for SINV in A. aegypti. Further studies are required to evaluate the occurrence of this phenomenon in nature using a variety of arboviruses and mosquito species.

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References


Feder ME, Cartano NV, Milos L, Krebs RA & Lindquist SL (1996) Effect of engineering Hsp70 copy number on Hsp70 expression and tolerance of ecologically relevant heat shock in


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